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TITLE OF THE INVENTION (280 characters max)

SCREENING METHODS AND COMPOSITIONS

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Respectfully submitted,

SIGNATURE _____ Date: 2/24/04

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[ ] Additional inventors are being named on separately numbered sheets attached hereto

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Applicant:)
Fernandez-Salas et al)
Serial No.: applied for)
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Enclosed herewith are the following documents:

(1) Return/postage paid Postcard

(2) Transmittal Sheet

(3) Provisional application Cover Sheet (1 pages)

(4) Specification (66 pages)

(5) Drawings (16 sheets)

Respectfully Submitted,

Bonnie Ferguson

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SCREENING METHODS AND COMPOSITIONS

Field of the Invention

The present invention is relevant to the fields of medicine, molecular biology, biochemistry and medicinal chemistry.

Background of the Invention

Neurotoxins, such as those obtained from *Clostridium botulinum* and *Clostridium tetanus*, are highly potent and specific poisons of neural cells. These Gram positive bacteria secrete two related but distinct toxins, each comprising two disulfide-linked amino acid chains: a light chain (L) of about 50 KDa and a heavy chain (H) of about 100 KDa, which are wholly responsible for the symptoms of these diseases. Proteolytic cleavage of the H chain gives rise to two fragments of approximately 50 KDa each: a carboxy terminal domain (H_C) which contains the cell surface-binding domain, and an amino terminal domain (H_N) which contains a region responsible for translocation of the toxin.

The tetanus and botulinum toxins are among the most lethal substances known to man, having a lethal dose in humans of between 0.1 ng and 1 ng per kilogram of body weight. Tonello et al., *Adv. Exp. Med. & Biol.*

389:251-260 (1996). Both toxins function by inhibiting neurotransmitter release in affected neurons. The tetanus neurotoxin (TeNT) acts mainly in the central nervous system, while botulinum neurotoxin (BoNT) acts at the neuromuscular junction by inhibiting acetylcholine release from the axon of the affected neuron into the synapse, resulting in a localized flaccid paralysis. The effect of intoxication on the affected neuron is long-lasting and has been thought to be irreversible.

The tetanus neurotoxin (TeNT) is known to exist in one immunologically distinct type; the botulinum neurotoxins (BoNT) are known to occur in seven different immunogenic types, termed BoNT/A through BoNT/G. While all of these types are produced by isolates of *C. botulinum*, two other species, *C. baratii* and *C. butyricum* also produce toxins similar to /F and /E, respectively. *See* e.g., Coffield et al., *The Site and Mechanism of Action of Botulinum Neurotoxin* in *Therapy with Botulinum Toxin* 3-13 (Jankovic J. & Hallett M. eds. 1994), the disclosure of which is incorporated herein by reference.

It is also recognized by those of skill in the art that within each type of clostridial neurotoxin there can be various strains varying somewhat in their amino acid sequence, and also in the nucleic acids encoding these proteins. Figures 16A-16C show an alignment of various known strains of BONT/A showing their amino

acid sequences and areas of difference. Additional BONT/A-producing strains are known in the art, such as, without limitation, strain NCTC 2012, and those listed in Franciosa et al., 32 J. Clin. Microbiol. 1911 (August 1994). BONT/A neurotoxin isolated form these strains may contain conservatively modified variations of their amino acid sequences and of the nucleotide sequences encoding them.

Regardless of type, the molecular mechanism of intoxication appears to be similar, although all details of this process are not yet precisely known and Applicants have no wish to be limited by the following description.

In the first step of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy chain and a cell surface receptor; this receptor is thought to be different for each type of botulinum toxin and for TeNT. The carboxy terminus of the toxin heavy chain appears to be required for targeting of the toxin to the cell surface.

In the second step, the toxin crosses the plasma membrane of the poisoned cell. The toxin is first engulfed by the cell through receptor-mediated endocytosis, and an endosome containing the toxin is formed. The catalytic domain of the toxin is then translocated from the endosome into the cytoplasm of

the cell. This last step is thought to be mediated by the amino terminus of the heavy chain, which undergoes a conformational change in response to a pH of about 5.5 or lower. Acidic endosomes are known to possess a proton pump which decreases intra-endosomal pH. The conformational shift is believed to expose hydrophobic residues in the translocation domain, which permits the toxin to embed itself in the endosomal membrane. The catalytic domain then translocates through the endosomal membrane, probably via a pore, into the cytosol.

The last step of the mechanism of botulinum toxin activity appears to involve reduction of the disulfide bond joining the heavy and light chain. The entire toxic activity of botulinum and tetanus toxins is contained in the light chain of the holotoxin; the light chain is a zinc (Zn++)-dependent endopeptidase.

In neurons, neurotransmitters are packaged within synaptic vesicles, formed within the cytoplasm, then transported to the inner plasma membrane where the vesicles dock and fuse with the plasma membrane. Recent studies of nerve cells employing clostridial neurotoxins as probes of membrane fusion have revealed that fusion of synaptic vesicles with the cell membrane in nerve cells depends upon the presence of specific proteins that are associated with either the vesicle or the target membrane. *See id.* These proteins have been termed SNAREs. A protein alternatively termed

synaptobrevin or VAMP (vesicle-associated membrane protein) is a vesicle-associated SNARE (v-SNARE). There are at least two isoforms of synaptobrevin; these two isoforms are differentially expressed in the mammalian central nervous system, and are selectively associated with synaptic vesicles in neurons and secretory organelles in neuroendocrine cells. The target membrane-associated SNAREs (t-SNARES) include syntaxin and SNAP-25. Following docking, the VAMP protein forms a core complex with syntaxin and SNAP-25; the formation of the core complex appears to be an essential step to membrane fusion. See Rizo & Sudhof, *id.* and Neimmann et al., *Trends in Cell Biol.* 4:179-185 (May 1994).

The endopeptidase of clostridial toxin light chain selectively cleaves SNARE proteins. TxNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G intoxication causes cleavage of VAMP. Most of the VAMP present at the cytosolic surface of the synaptic vesicle is removed as a result of any one of these cleavage events. Each toxin specifically cleaves a different bond and/or substrate, except TeNT and BoNT/B, which cleave the same bond in the VAMP protein.

BoNT/A and /E selectively cleave the plasma membrane-associated protein SNAP-25; this protein is bound to and present on the cytosolic surface of the plasma membrane. BoNT/E cleaves a 26-amino acid fragment from the C terminus of SNAP-25 whereas BoNT/A

removes only nine residues, creating a 197-amino acid fragment ($SNAP25_{197}$) that is 95% the length of SNAP-25. BoNT/C cleaves syntaxin, an integral protein having most of its mass exposed to the cytosol. Syntaxin interacts with the calcium channels at presynaptic terminal active zones. *See* Tonello et al., *Tetanus and Botulism Neurotoxins in Intracellular Protein Catabolism* 251-260 (Suzuki K & Bond J. eds. 1996). BoNT/C_1 also cleaves SNAP-25 at a peptide bond next to that cleaved by BoNT/A.

Both TeNT and BoNT are taken up at the neuromuscular junction. BoNT remains within peripheral neurons, and blocks release of the neurotransmitter acetylcholine from these cells. Through its receptor, TeNT enters vesicles that move in a retrograde manner along the axon to the soma, and is discharged into the intersynaptic space between motor neurons and the inhibitory neurons of the spinal cord. At this point, TeNT binds receptors of the inhibitory neurons, is again internalized, and the light chain enters the cytosol to block the release of the inhibitory neurotransmitters 4-aminobutyric acid (GABA) and glycine from these cells. Id.

Because of its specifically localized effects, dilute preparations of BoNT have been used since 1981 as therapeutic agents in the treatment of patients having various spastic conditions, including strabismus (misalignment of the eye), blepharospasm (involuntary

eyelid closure) and hemifacial spasm. *See* e.g., Borrodic et al., *Pharmacology and Histology Botulinum Toxin* in *Therapy with Botulinum Toxin* 3-13 (Jankovic J. & Hallett M. eds. 1994). Of the seven toxin types, BoNT/A is the most potent of the BoNTs, and the most well characterized.

Intramuscular injection of spastic tissue with dilute preparations of BoNT/A has also been used effectively to treat spasticity due to brain injury, spinal cord injury, stroke, multiple sclerosis and cerebral palsy. The extent of paralysis depends on both the dose and dose volume delivered to the target site. Typically, the neurotoxin is administered in a preparation that also contains several non-toxic proteins as well, including hemagglutins and associated glycoproteins that assist in maximizing its stability and presentation to the target motor neuron.

Recently, it has been suggested that BONT's and TeNT bind neurons with through a high-affinity interaction between the toxin binding domain (Hc), a ganglioside, and an unknown protein receptor at the nerve terminal. See e.g., Schiavo et al., 80 *Physiol. Rev.* 717 (2000), Williamson, 274 *J. Biol. Chem.* 25173 (1999), Chaddock, *Clostridium Botulinum And Associated Neurotoxins* in *Molecular Medical Microbiology* 1141 (Academic Press 2001). Although toxin binding is not strictly dependent on the presence of gangliosides, the presence of specific gangliosides appears to be

required for high affinity binding. In particular, BONTs have been observed to interact *in vitro* and *in vivo* with polysialogangliosides, especially those of the G1b series (GD1b, GT1b and Gq1b) Halpern & Neale, 195 *Curr. Topics Microbiol.* 221 (1995).

A Japanese group and a research group from the University of Wisconsin have reported the results of experiments that suggest the identification of a neural cell membrane receptor for BONT/B and the involvement of ganglioside GT1b in binding of this neurotoxin to its target. See Kozaki S., 25 *Microb. Pathog.* 91-99 (Aug. 1998); Dong, M. et al., *J. Cell Biol.* 62:1293(September 29, 2003). While these results appear to show that fragments of synaptotagmin I and synaptotagmin II bind BONT/B, the results are largely circumstantial. Indeed, there is later evidence which intimates that these proteins may simply be chaperone proteins for the neurotoxin. Coffield and Kalandakanond, Abstract 29 page R16.

Thus, to date the identity of the membrane receptor for any of the clostridial neurotoxins has not been conclusively demonstrated.

Summary of the Invention

The present invention is based on the identification of a neural cell membrane receptor to which the BONT/A neurotoxin selectively binds as the

first step to the selective intoxication of the neuron. The present inventors have also identified specific gangliosides which facilitate binding of BONT/A to the FGFR3 receptor and the internalization of the BONT/A and BONT/E neurotoxins within the neural cell.

All of the publications cited in this application are hereby incorporated by reference herein.

Brief Description of the Drawings

Figure 1 is a graph showing the decrease in insulin secretion from HIT-TI5 cells given PURE-A toxin by electroporation. As can be seen, the addition of glucose to 25 mM induced insulin secretion from untreated cells and cells subjected to electroporation without the addition of PURE-A. However, cells into which PURE-A was introduced were unresponsive to induction of insulin secretion.

Figure 2A demonstrates that the presence of the toxin delayed growth in HIT-T15 cells when compared to controls, but toxin-treated cells were able to replicate normally after a recovery period. Figures 2B and 2C demonstrate that cleavage of SNAP-25 was detected by Western blot at all time points tested when PURE-A was introduced into the cells.

Figure 3 shows HIT-T15 cells, transformed with a human brain cDNA library and selected using magnetic beads to which BONT/A had been bound. Individual colonies are visible in the dish and are surrounded by magnetic beads.

Figure 4 shows the results of an assay of insulin release from HIT-T15 cells containing the putative BONT/A receptor. Cells were exposed to 1 nM PURE-A and inhibition of insulin release upon glucose stimulation was measured using an enzyme-linked immunoassay.

Figure 5 shows the reduction of insulin release in representative HIT-T15 transformants C6 and C7 upon incubation with BONT/A.

Figure 6 shows Western blots of clones C6 and C7 incubated with BONT/A using anti SNAP-25 antibody.

Figure 7A shows the results of a PCR amplification of a region bordering the human brain library cDNA insert in the c7 cell line.

Figure 7B shows a further amplification of from the C7 cDBNA primer using a set of nested primers.

Figure 8 shows the levels of SNAP-25_{197} produced upon incubation of three cell lines (Neuro-2A, SH-SY5Y, and NG108-15) with BONT/A.

Figure 9A shows the time course of the appearance of the $SNAP_{197}$ fragment in Western blots of Neuro 2 cell lysates following BONT/A treatment. Figure 9B shows the same experiment in SH-SY5Y cells.

Figure 10 shows the cooperative effect of ganglioside GT1b on the rate and/or extent of SNAP-25 cleavage in Neuro-2A cells treated with 12.5, 25 and 50 pM BONT/A.

Figure 11 the overall reaction scheme to crosslink BONT/A with the putative receptor in Neuro-2A cells using the cross-linking reagent sulfo-BED.

Figure 12A illustrates the isolation of a complex of approximately 250 kDa from Neuro-2A cells containing the 150 kDa neurotoxin cross-linked to the putative BONT/A receptor. Bands were visualized with silver staining.

Figure 12B shows the same sample, electrophoresed on a reducing gel to separate toxin from receptor, then transferred to a membrane for Western blotting. Subsequent probing of the membrane with hyperimmune neutralizing serum to PURE A results in two bands: one corresponding to the holotoxin (150 kDa) and the other to heavy chain (100 kDa). In this Figure, the stronger binding of the serum to the heavy chain in preference

to the light chain results in preferential detection of the heavy chain rather than the light chain.

Figures 13A-D shows a Western blot of the membrane-transferred proteins from the reducing gel with antibodies selective for each of FGFR1, FGFR2, FGFR3, and FGFR4, respectively. Only antibodies selective for FGFR3 (Figure 13C) bound to bands in the Western blot.

Figure 14 A-D shows Western blots taken from lysates from Neuro-2A, SH-SY5Y, PC-12, HIT-T15, and HIT-T15 clone C7 subjected to SDS-PAGE. The membrane-transferred proteins were evaluated for the presence of FGFR1-4 using antibodies selective for each of these FGF receptors (Figures 14 A-D, respectively).

Figure 15 shows the results of a Western blot of a receptor competition experiment in Neuro-2 cells of PURE-A and FGF.

Figure 16A-C shows an alignment of some representative strains of BONT/A.

Detailed Description of the Invention

The present invention is based in part on the finding that high affinity binding of neurotoxins, particularly (though not exclusively) BONT/A, to neuronal cells requires a ganglioside component and a

protein receptor component. To this end, the present inventors have first discovered that the fibroblast growth factor 3 (FGF3) receptor, when, is sufficient to bind and direct the intracellular internalization of BONT/A. Secondly, the inventors have discovered that when the FGFR3 is combined with gangliosides, particularly the ganglioside GT1b, the affinity of both toxin binding and internalization is significantly increased.

The C-terminal region of the heavy chain of BONT/A (termed the Hc region), like all known clostridial neurotoxins, is involved in specific binding to a neuronal receptor present on the external surface of neurons.

The Hc binding domain is composed of two distinct structural domains (reviewed in Poli, M.A. and Lebeda F.J., *An overview of Clostridial Neurotoxins* in I *Handbook of Neurotoxicology* 293-304 (E.J. Massaro ed., Humana Press, 2002), and Meunier et al., *Molecular mechanism of action of botulinal neurotoxins and the synaptic remodeling they induce in vivo at the skeletal neuromuscular junction* in I *Handbook of Neurotoxicology* 305-347 (E.J. Massaro ed., Humana Press, 2002). The N-terminus of the Hc region presents a jelly-roll architecture related to that of the S-lectins, a carbohydrate-binding family of proteins. By contrast, the C-terminus of Hc is in a pseudo threefold trefoil conformation that presents structural similarity to the

sequentially unrelated interleukins-1α and 1β, Kunitz-type trypsin inhibitors, as well as fibroblast growth factors (FGF). These proteins, mostly β-proteins, are involved in protein-protein interactions. This analysis is therefore consistent with the hypothesis that clostridial neurotoxins bind to receptors comprised of two components; a protein component and a carbohydrate component.

The amino acid sequence at the C-terminus of Hc is poorly conserved among different clostridial neurotoxins, and competition experiments have shown that different BoNT serotypes bind to different protein receptors on the surface of neuronal cells. Further, BoNTs are known to interact *in vivo* and *in vitro* with polysialogangliosides, in particular with members of the G1b series (GD1b, GT1b, and Gq1b) (reviewed in Halpern and Neale, 195 *Current Topics Microbiol. Immunol.*, 221-241 (1995). Preincubation of the toxin with these gangliosides protects the neuromuscular junction (NMJ) of mice from BoNT toxicity.

High-affinity, trypsin-sensitive, BoNT-binding sites were found in isolated synaptosomes (Williams et al, 131 *Eur. J. Biochem.* 437-445 (1983)). Since lectins with high affinity for sialic acid antagonize the binding of BoNTs, their protein receptors may be glycoproteins. Receptors for BoNTs would direct them to acidic vesicles allowing the translocation of the LC into the cytosol of the neuron.

Based on these findings, and as the result of the data described herein, the Applicants have discovered that the fibroblast growth factor receptor 3 (FGFR3) binds BONT/A on the surface of neural cell lines. Internalization of the toxin can be followed when these cell lines are exposed to the toxin. Moreover, BONT/A internalization is inhibited in a dose-dependent manner when FGF, preferably an FGF selected from FGF 1, 2, and 9, is added at increasing concentrations. Cells tested by the Applicants that did not display the FGFR3 receptor were unable to internalize the toxin, although when subjected to electroporation in the presence of BONT/A, the intracellular cleavage of SNAP-25 could be detected, indicating that the endopeptidase activity of the toxin remained intact, and that the cells remained susceptible to the endopeptidase.

Accordingly, in one embodiment the present invention is directed to a method of screening a sample for the presence of a molecule able to compete with BONT/A for selective binding to neural cells of the neural muscular junction comprising:

a) contacting said sample with a composition comprising FGFR3 receptor and, optionally, GT1b ganglioside, and

b) determining whether said molecule selectively binds FGFR3,

wherein selective binding of said molecule to FGFR3 indicates that said molecule is able to inhibit or reduce BONT/A toxicity to said neural cells.

By "BONT/A" or "type A" or similar terminology referring unambiguously to *Clostridium botulinum* neurotoxin type A is meant any of a number of polypeptide neurotoxins, and derivatives thereof, which can be purified from *Clostridium botulinum* serotype A strains and which share FGFR3 as a cell surface receptor. Such neurotoxins include those found in or corresponding to the following strains and accession numbers:

Strain	accession numbers
CL138	AAQ16535
137	AAQ16534
129	AAQ16533
13	AAQ16532
42N	AAQ16531
Hall A-hyper	AAM75961
667Ab	CAA61124
NCTC 2916, P10845	CAA36289
Allergan-Hall A	AAQ06331
62A	AAA23262
Kyoto-F	CAA51824
type A NIH	BAA11051
NCTC 7272	
7I03-H	
Kumgo	AAO21363

Additionally, BONT/A includes those polypeptides whose amino acid sequences are listed in Figure 16 hereof.

The molecule to be tested in the screening method may be a "small" organic compound of synthetic origin, or may be a macromolecule (either of synthetic or biological origin), such as a polypeptide (including

without limitation, a growth factor, a neurotoxin, a modified neurotoxin, an antibody or an antibody derivative), nucleic acid (such as a nucleic acid aptomer), or polysaccharide (such as a ganglioside or a lectin). In one preferred embodiment, the molecule is a synthetic molecule designed based on the tertiary structure and three dimensional conformation of FGF or an antibody that inhibits BONT/A binding to the FGF receptor. Such SAR (structure/activity relationship) analysis is routine in the art of medicinal chemistry, among other fields.

Determination of whether the molecule selectively binds the FGFR3 can be accomplished in any appropriate manner. For example, and without limitation, the molecule may be labeled with a detectable moiety, such as a radionuclide (e.g., ^{14}C, ^{3}H, ^{32}P, ^{35}S or the like), a fluorescent moiety, or with an affinity tag, such as poly histadine, biotin, or GST (glutathione-S-transferase). The latter tags, which are common in the art and can be created as part of a fusion protein, can be used to purify the receptor/test compound complex.

By "selective" binding is mean that a binding agent is able to bind its target under physiological conditions, or in vitro conditions substantially approximating physiological conditions, to a statistically significantly greater degree (i.e., has a smaller K_d or dissociation constant) than to other, non-target molecules on the surface of the neural cell.

"K_d" is the molar concentration of the binding agent at which half the target molecules are bound by the binding agent.

Alternatively, the binding of the molecule and receptor can be detected using labeled or unlabeled antibodies selective for (as an example) the molecule (test compound), FGFR3, or the ganglioside (such as GT1b). If the antibody is labeled, the binding of the molecule can be detected by various means, including Western blotting, direct microscopic observation of the cellular location of the antibody, measurement of cell or substrate-bound antibody following a wash step, or electrophoresis, employing techniques well-known to those of skill in the art. If the antibody is unlabeled, one may employ a labeled secondary antibody for indirect detection of the bound molecule, and detection can proceed as for a labeled antibody.

Other means of detecting the binding of the molecule include competition experiments, whereby the molecule is used to test for inhibition of FGF or BONT/A binding to the FGFR3, either directly or indirectly. Indirect means may include detection of the effect of the molecule (test compound) on the activity of FGF or BONT/A, for example, by detecting the cleavage of full-length SNAP-25 to SNAP-25_{197} by the neurotoxin.

In another embodiment, the invention is drawn to a polypeptide comprising at least the H_c region of BONT/A, which is produced from a bulk or formulated preparation wherein the bulk or formulated preparation is assayed for specific binding to neural cells using a method comprising contacting said polypeptide with a composition comprising FGFR3 receptor and, optionally, GT1b ganglioside, and determining whether said polypeptide selectively binds FGFR3.

In another embodiment similar to the above aspect of the invention, the polypeptide comprises at least an FGFR3 binding domain, other than the H_c domain of BONT/A. Such a binding domain may comprise, for example, an FGF, such as FGF 1, FGF2, FGF4 or FGF 9, or an anti-FGFR3 antibody. Further, the polypeptide may optionally contain a translocation domain such as the H_N domain of BONT/A. Additionally, the polypeptide will generally contain a clostridial neurotoxin light chain or variation thereof - the nature and/or source of the light chain can provide differences in the extent and half-life of the therapeutic effect of the polypeptide.

Thus, in this embodiment the claimed polypeptide is produced (which production may include purification, enzymatic treatment, and/or oxidation steps) from a bulk or formulation preparation. In one preferred embodiment the preparation may be, for example, a cell lysate from fermentation of a BONT/A-producing strain

of Clostridium botulinum, or from a suitable mammalian, insect or bacterial host cell producing a recombinant version of BONT/A. Such a bulk preparation may also be produced using cell-free transcription methodologies. In another embodiment the preparation may be purified BONT/A formulated with associated stabilizing proteins, such as serum albumin. In each case, the preparation may comprise BONT/A molecules which are denatured or otherwise incorrectly folded so as not to bind to the target cells. The potency and/or specific activity of the preparation, or of fractions purified from the preparation, can be detected by using the claimed assay method.

Alternatively, the polypeptide to be assayed may comprise only a portion of the entire BONT/A molecule. For example, the bulk preparation may contain only the heavy chain of BONT/A, as separate production of the heavy and light chains of the toxin may be a preferred way of avoiding accidental exposure to the neurotoxin by laboratory workers.

As another example of the above embodiment, the polypeptide may comprise a chimeric recombinant polypeptide which contains the Hc region of the heavy chain of BONT/A (or some other FGFR3-binding moiety, such as FGF itself). The chimeric polypeptide comprises amino acid sequence regions additional to, or other than, those present in the wild-type BONT/A BONT/A molecule. For example, as disclosed in US

Patent No. 6,203,794, hereby incorporated by reference herein, botulinum and tetanus toxins may be used as the basis for the creation of transport proteins. The light chain of these transport proteins are generally either replaced by a therapeutic moiety or inactivated and coupled to such a therapeutic moiety. Additionally, International Patent Application WO0114570, published March 1, 2001, discloses chimeric neurotoxins comprising polypeptides containing domains of more than one neurotoxin. Thus, this aspect of the invention also encompasses, as a preferred embodiment, chimeric neurotoxins containing at least the Hc domain of BONT/A. Such molecules may be useful in modulating the time or extent of the inhibition of secretory vesicle release. Further, it may be desirable to target agents, such as therapeutic agents, to the extracellular surface of the neural cell membrane. Thus, such an agent may be joined (e.g., as a fusion protein or via post translational conjugation) to the H_c portion of BONT/A. In such a case the cell lysate or conjugation reaction mixture may comprise a batch preparation in accordance with this aspect of the invention.

The above-referenced polypeptides are screened for binding and/or internalization essentially as mentioned above in the described screening method embodiment.

In yet another embodiment, the present invention is drawn to a method of marketing a polypeptide which

contains a region capable of binding the FGFR3 receptor comprising:

a) obtaining permission from a governmental or regional drug regulatory authority to sell said polypeptide, wherein said polypeptide is first produced from a bulk preparation which is assayed for selective binding of said polypeptide to neural cells by contacting the bulk preparation containing said polypeptide with a composition comprising FGFR3 receptor, and optionally GT1b ganglioside, and determining whether said polypeptide selectively binds FGFR3 under such conditions,

b) packaging said polypeptide for sale in a manner consistent with the requirements of said regulatory authority, and

c) offering said polypeptide for sale.

In this embodiment the invention is drawn to a method of marketing a polypeptide containing the H_c region of a BONT/A toxin. The polypeptide at issue in this embodiment of the invention is produced from a bulk preparation which is assayed for purity or activity using the screening method described previously. In a step of this method, permission is obtained from a regulatory body for the marketing of such polypeptide. In this context "permission" may be

tacit or express; that is, permission or approval may be obtained from the regulatory authority for the sale of a therapeutic agent or composition comprising said polypeptide, in which case "permission" is marketing approval for the sale of such agent or composition. Alternatively, "permission", as used herein, may comprise the assent, either affirmatively given or manifested by its lack of objection, of such regulatory authority to the continued sale of a product containing a polypeptide assayed in this new manner. As before, the polypeptide may comprise BONT/A, or a derivative thereof, or a fusion protein or conjugate containing the H_c region of the BONT/A heavy chain.

By a "sample" or "preparation" is meant a preparation containing a agent or compound to be tested for its ability to bind to the BONT/A receptor. As used herein, a sample includes, without limitation, a solution comprising a small molecule, an antibody preparation, a cell lysate, a cell culture medium, a bulk or formulation preparation of a therapeutic agent, or a fraction from a purification process.

By an "agent" able to compete with BONT/A for selective cell binding is meant a compound, molecule, multimer, complex, or other composition which has the same cell surface target as BONT/A.

By "LD_{50} assay" is meant an *in vivo* assay of neurotoxin activity comprising determining the dose of neurotoxin at which 50% of treated animals die.

Unless the context clearly indicates otherwise, by a "polypeptide", is meant a composition comprising one or more associated polymeric chains of amino acids linked by peptide bonds. If comprised more than one chain, the chains may be linked or otherwise associated by interactions such as disulfide bonds or ionic or hydrophobic interactions.

The therapeutic product comprising the polypeptide originally contained in the bulk preparation so assayed is labeled in accordance with the requirements of the regulatory authority. The product is then offered for sale. Offering for sale may comprise advertising or sales activity, educational seminars directed at doctors, hospitals, insurers, or patients, conversations with state, regional or governmental officials concerning subsidy reimbursement (such as Medicare or Medical).

Identification of BONT/A Receptor and Examples

We utilized two approaches for the identification of BoNT/A receptor: a) genetic complementation, and b) isolation from cells able to take up the BONT/A toxin with high affinity.

Cloning by genetic complementation is achieved by transfecting cells that do not contain the receptor with a vector containing a retroviral cDNA library constructed from a tissue known to express the receptor. Selection of cells expressing the receptor from a pool of transfected cells can be achieved by several means, such as binding the selected protein, or measuring its biological effect. According to this strategy, the receptor is then identified by PCR cloning of cDNA obtained from cells expressing the receptor.

The second approach, isolation of the putative receptor from cells known to expressing the receptor, required the identification of one or more cell line expressing a high affinity BONT/A receptor. Once identified the bound toxin is cross-linked with the receptor *in situ* and the cross-linked complex is isolated. Identification of the putative receptor can then be achieved by any number of means, including (without limitation) liquid chromatography-mass spectroscopy (LC-MS), or by Western blots using antibodies selected based on information on the literature which might offer clues as to the identity of the putative receptor.

Using both approaches we have identified FGFR3 as a receptor system enabling BoNT/A to selectively bind and enter neurons and other BONT/A susceptible cells.

Since the conformation of the binding domain of the various BoNT serotypes are structurally very similar (although the precise amino acid sequences vary) we also believe that serotypes other than BoNT/A may be able to use other members of the FGFR family (or other closely related members of the tyrosine kinase receptor superfamily, such as, without limitation, FGFR1, 2, 4 or 5 the PDGF receptor, and the EGF and VEGF receptors (including any isoforms) as a receptor in the plasma membrane of neuronal and other susceptible cells. Such other cells include chromaffin cells, acinar cells, and insulinoma cells. *See* Lomneth, 18 *J. Toxicol. Toxin Rev.* 77-94 (1999).

I. Cloning of BoNT/A receptor by Genetic Complementation

We first undertook the cloning of the neurotoxin receptor by genetic complementation. This procedure involves transferring the DNA for the receptor to a cell line that does not contain the receptor naturally. For this latter purpose we choose the insulinoma HIT-T15 cells. HIT-T15 cells secrete insulin in response to high levels of glucose in the medium, however BONT is unable to bind to these cells; thus extracellular exposure of HIT-T15 cells to BONT does not effect the secretion of glucose.

Despite the fact that HIT-T15 cells are normally unaffected by BONT, the intracellular secretory

exocytic process of these cells depends on the presence of SNAREs (which are targets of the neurotoxin endopeptidase activity) for vesicle docking and fusion. Specifically, HIT-T15 cells contain SNAP25, and intracellular introduction of BoNT/A by means not dependent upon toxin binding (such as permeabilization or electroporation) results in cleavage of the 206 amino acid full length SNAP25 molecule ($SNAP25_{206}$ into $SNAP25_{197}$, the BONT/A specific cleavage product, resulting in failure of vesicle docking at the intracellular membrane surface, and the inhibition of insulin release.

Since neurons of the central nervous system are known to bind and internalize BONT/A, we used a human brain retroviral DNA library (purchased from Clontech, Inc.) as a source of the BONT/A receptor cDNA.

a) Receptor Cloning in HIT-T15 Cells

We packed the library into viruses using the Amphopack® 293 producer cell line. The retroviral library was then used to infect HIT-T15 cells. Cells assumed to be expressing the receptor clones were selected based on their ability to bind Dynex magnetic beads coated first with an antibody against the light chain of BONT/A, and then with pure BONT/A (PURE A); in this way the neurotoxin molecules were correctly oriented with the Hc (receptor binding domain) domain pointed outward.

Cells binding to the magnetic beads were isolated using a magnet. The cells were then plated in culture media and colonies permitted to develop in the dishes. Individual colonies were picked and expanded to a 24 well plate to establish cell cultures. The isolated cells were grown and exposed to 1nM Pure BONT/A for 24 hours. Putative cell lines containing the receptor were selected based on the detection of SNAP-25 $_{197}$ in Western blot analysis (using anti-SNAP25 $_{197}$ antibody) of whole cell lysates, and by the detection of the inhibition of insulin release.

Genomic DNA from cells testing positive for cleavage of SNAP25 or inhibition of insulin release was isolated and used in PCR reactions using the Clontech ADVANTAGE® Genomic PCR kit and the following primers (written from 5' to 3'):

AGCCCTCACTCCTTCTCTAG
SEQ ID No: 1

ACCTACAGGTGGGGTCTTTCATTCCC
SEQ ID NO: 2

to amplify and isolate the human receptor gene present in the cells. Conditions were as recommended using the Clontech ADVANTAGE® Genomic PCR kit. Reactions were incubated at 95 degrees Celsius for 1 minute, followed by 25 cycles at 68 degrees C for 30 seconds and 95

degrees C for 30 seconds, followed by 1 cycle at 68 degrees C for 6 minutes and final incubation at 4 degrees C.

A 12 kb fragment of DNA was identified and purified from the PCR reaction and subjected to a second PCR amplification. The primers used in the second PCR were nested primers designed to anneal to sequences of DNA within the amplicon originally produced, and had the following nucleotide sequences.

CCCTGGGTCAAGCCCTTTGTACACC

SEQ ID NO: 3

TGCCAAACCTACAGGTGGGGTCTTT

SEQ ID NO: 4

b) Inhibition of Insulin Secretion in HIT-T15 cells

The effect of BONT/A treatment on exocytosis was analyzed in HIT-T15 insulinoma cell transfectants selected as indicated above, and suspected to express the BONT/A receptor. These cells were treated for 15 hours with 1nM pure BONT/A. Insulin secretion was measured in DMEM containing high glucose (25mM) or low glucose (5.6 mM). After 1 hour incubation with high glucose media at 37°C, the amount of insulin in the media was determined using an Insulin ELISA kit from Peninsula Laboratories. Exocytosis is expressed as the amount of insulin secreted per 1 X 10^5 cells per hour.

II) Isolation Of The Putative Receptor From Cells Known To Express The BONT/A Receptor

a) Immunostaining PC12, HIT-T15, and Neuro-2 cells for Ganglioside GT-1b

Immunocytochemistry was performed on PC-12 (rat phecochromacytoma cells), Neuro-2 (mouse neuroblastoma), and HIT-T15 cell lines. These cells were fixed and probed with primary antibodies specific to the ganglioside GT-1b (obtained from Seikagaku Corp.) that is thought to be important for toxin uptake. We used Alexa®-fluor 488 (Molecular Probes, Inc.), conjugated secondary antibodies to visualize the GT-Ib via fluorescent microscopy. Those of skill in the art will recognize that Alexa®-fluor may be substituted with any fluorophore of similar intensity.

b) Identification of cell lines with high affinity uptake for BoNT/A

A method for neurotoxin treatment followed by Western Blot was developed to screen cell lines for toxin uptake using an antibody to detect the BONT/A cleavage product $SNAP25_{197}$. Tested cell lines (human unless indicated otherwise) were: Neuro 2A, SH-SY%Y, NG108-15, N1E-115, SK-N-BE(2), SK-N-DZ, SK-N-F1, BE(2)-C, SK-N-SH, NB4 1A3, BE(2)-M17, C1300, NG108-15, HCN-1A, HCN-2 TE 189.T, ND8/34.

Cells of each candidate cell line were plated in poly-D-lysine/Laminin coated 6-well plates for 24 hours. BoNT/A (900 KDa complex) was added to the cells at different concentrations (as described in the figures and the results section below) in the culture medium overnight. Cells were then collected in 15 ml Falcon tubes, washed once with 1ml of PBS, and then transferred to 1.5 ml microcentrifuge tubes. Cells were lysed in 0.5 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10% glycerol and 1% Triton X-100®) on a rotator at 4°C for 1 hour. Lysed cells were centrifuged at 5000 rpm at 4°C for 10 minutes to eliminate debris; supernatants were transferred to fresh siliconized tubes.

Protein concentrations were measured by Bradford's method and resuspended in 1 x SDS sample buffer at 1mg/ml or higher concentration. Samples were boiled for 5 min, 40 µl of the cell-free lysates were loaded on 4-12% Tris-HCl polyacrylamide gradient gels. The separated proteins were transferred to PVDF membranes for Western analysis and the membranes blocked in 5% non-fat milk in TBST buffer for 1 hour at room temperature. The cleaved $SNAP25_{197}$ was detected with anti$SNAP25_{197}$ antibody diluted in blocking buffer; blot was washed extensively, and the bound antibody was detected with a horseradish peroxidase-conjugated species-specific secondary antibody.

The Typhoon 9410 Imager (Amersham) for Western Blot Analysis was used instead of traditional film. After the final washes the membrane was reacted with ECL Plus Western blot detection reagent (Amersham), membrane was then incubated at room temperature for 5 min in order for the images to develop. The choice of pixel size and PMT voltage settings depended on each individual blot. Membranes were scanned and quantified using Typhoon Scanner and Imager Analysis software.

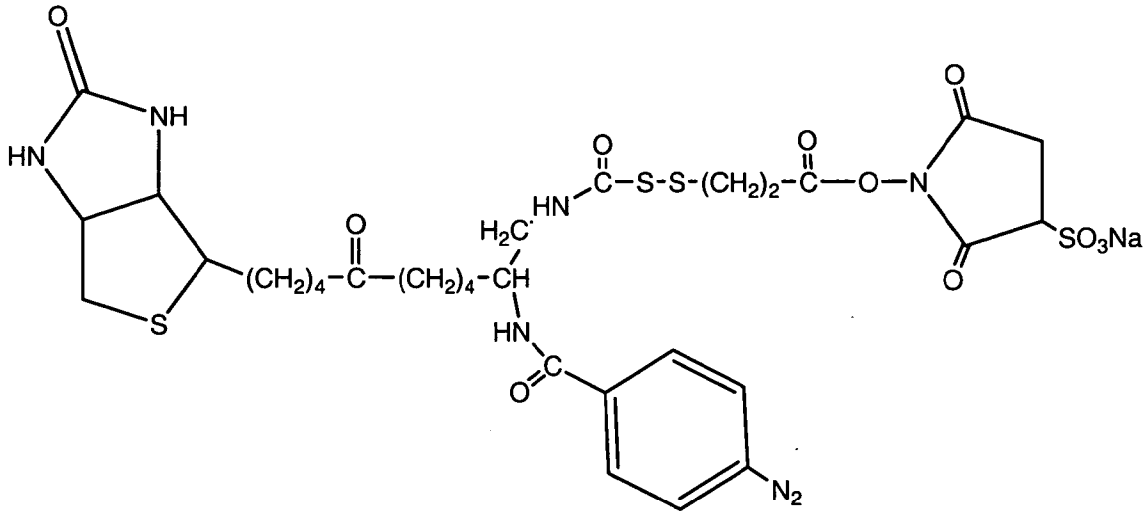
c) GT1b Ganglioside Treatment

Cells were cultured in Poly-D-lysine/Laminin coated 6-well plates in culture medium with 10% FCS for 24 hours. GT1b ganglioside (Alexis, San Diego, CA) was dissolved in PBS at 5mg/ml as a stock solution and stored at -20°C. At the time of treatment, GT1b was diluted in serum free medium to a final concentration of 25µg/ml and added to the cells in culture. The cells were incubated at 37°C, under 7.5% CO_2 for 24 hours. Following incubation, the GT1b-containing medium was removed and 0.5 ml to 5 ml BoNT/A complex was added at various concentrations (ranging from 0.125 nM to 5 nM) in culture medium (as recommended by the ATCC or ECACC tissue collections for that cell line) containing 10% FBS and incubated overnight.

d) Purification of the BoNT/A receptor from Neuro-2A Cells

Neuro-2 cells were found to have the most rapid toxin uptake profile (about 10 minutes) and affinity to the toxin, and were thus chosen for further study based on the presence of a high affinity toxin uptake system. Neuro-2 cells were therefore exposed to 5nM PURE-A at 4°C for 2-4 hours and subsequently treated with one of the two cross-linking agents BS^3 and sulfo-SBED (Pierce). The reagent BS^3 is an 11.4-Å chain water-soluble and membrane-impermeable agent that is not cleaved by reducing agents. The reagent sulfo-SBED contains three reactive groups (one of them designed to be UV-activated) and is designed to biotinylate a target protein. Its structure (including the presence of a biotin molecule) is given below:

STRUCTURE OF SULFO-SBED



The procedure for cross-linking the receptor and PURE-A was as follows.

All reactions are performed at 4 degrees C. 100 ug of Pure A 0.34mL) is pelleted at 10,000 x g for 10 minutes, and brought up in a final volume of 900 ul PBS (pH 7.4). The reaction is then transferred to the dark. Ten milligrams of Sulfo-SBED reagent is dissolved in a volume of 454 ul DMSO, to make a solution of 25 mM SBED. This is immediately serially diluted as follows: 100 ul of the 25 mM SBED reagent is added to 0.9 mL PBS (2.5 mM SBED)/ 10% DMSO). This in turn is diluted in the same manner to yield a 0.25 mM SBED)/ 1 % DMSO solution.

One hundred microliters of this latter solution is added to 900ul PURE A in PBS to yield 0.67 uM PURE A, 25 uM Sulfo-SBED, 0.1% DMSO in 1 ml, and incubated in a 4°C incubator for two hours in a secondary container on shaking apparatus. The reaction is stopped by the addition of 50 ul 1M TRIS pH 7.4 (final concentration 50mM). The solution is inverted 6 times and allowed to incubate 30 minutes on ice. Meanwhile, Neuro-2A cells are harvested by centrifugation and washed 3 X with cold TBS, then divided into two aliquots of 4 X 10^8 cells. Each aliquot of cells is suspended in 12 mL cold TBS, and placed on ice for 15 minutes (3X10^7 cells/mL). One milliliter of the Sulfo-SBED/PURE A solution is added to one of the Neuro-2A cell

suspension aliquots. The other aliquot receives the sulfo-SBED only (as a control). The final dilution thus contains 4 X 10^8 cells (2 X 10^7 cells/mL) and 100 ug PURE A (33nM) in a 20 mL volume.

The PURE A/SBED and neuro-2 cell suspension is incubated at 4°C in an incubator for two hours in a secondary container on shaking apparatus. Each cell solution is distributed in 13 aliquots of 1.0 mL. These aliquots are exposed to ultraviolet radiation (365 nm) for 15 minutes. The cells are harvested by centrifugation, washed with cold TBS, and lysed overnight at 4°C with 0.4 ml TRITON X-100 lysis buffer (50mM Hepes, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1% TRITON X-100, and suitable amounts of a protease inhibitor).

Following lysis, each aliquot is centrifuged at 5000 x g to remove unbroken cells, then the lysates each given 0.05mL of avidin-beads and incubated for 3 hours at 4°C. The avidin beads are then washed twice with 0.5mL lysis buffer (centrifuge at 1000 x g to pellet beads) followed by two washed with 0.5mL PBS buffer. The supernatant is then removed and combined with 100 ul SDS-PAGE loading buffer, then subjected to SDS-polyacrylamide gel electrophoresis.

e) Anti-FGFR antibodies used in Western blots

Antibodies to the cytoplasmic region of the polypeptides FGF 1 receptor (FGFR1), FGF 2 receptor (FGFR2), FGF 3 receptor (FGFR3) and FGF 4 receptor (FGFR4) were purchased from Santa Cruz Biotechnologies. The precipitated receptor-PureA complex (obtained as described above) was electrophoresed in an SDS polyacrylamide gel under reducing and non-reducing conditions. The resulting proteins were Western blotted and probed with the antibodies to FGFR1-FGFR4. Western blots were also prepared of cell lysates from Neuro-2A, HIT-T15 cells, HIT-T15C7 (i.e., the selected clone constructed as mentioned above and containing the putative receptor), SH-SY5Y, and PC12 cells to study the expression of the FGFR in the cell lines.

f) FGF-Pure A Competition Experiments

Neuro-2A cells were plated at a density of $5x10^5$ cells/well in 6 well plates the day before the experiment. PURE-A was added to the culture media at a final concentration of 5nM, either alone or in combination with FGF1, FGF2 or FGF1+FGF2 at various concentrations ranging from 0.1nM (physiological concentration for FGF) to 200nM. The treated cells were then incubated at 37°C for 10 min with the mixture. Cells were thereafter lysed and subjected to Western blot using an antibody to SNAP25 (SMI-81, from Sternburger Monoclonals) that recognizes both uncleaved and cleaved SNAP25.

RESULTS

Strategy 1: Genetic Complementation

The insulinoma cell line HIT-T15 secretes insulin upon glucose stimulation, depending on the presence of intact SNAP25. We decided to use this cell line for the genetic complementation because it permits the use of two separate indications of neurotoxin activity: Western blotting to detect the cleaved SNAP-25 ($SNAP25_{197}$), and the detection of insulin secretion by monitoring insulin in the extracellular medium.

PURE-A was introduced into HIT-T15 cells by electroporation using a BioRad GENE PULSER® II to assess the cells' response by both parameters listed above before the receptor cloning started. Figure 1 shows that the presence of toxin inhibited exocytosis of insulin from cells induced to secrete insulin by the addition of 25 mM glucose.

Figure 2A shows the growth of HIT-T15 cells which were electroporated without and with PURE-A and their growth monitored for 10 days. Electroporation in the presence of PURE-A caused reduction in the cell number few days after cells were treated, but following this the growth of the cells was comparable to the control cells. Presence of toxin in the cells was followed by antibody detection of $SNAP25_{197}$ in the samples (C: control, E: electroporated without toxin, PA: electroporated with PURE-A). The antibody to SNAP25 in

Figure 2B detects cleaved and uncleaved products, while the antibody used in Figure 2C detects only $SNAP25_{197}$.

To evaluate if the presence of the toxin in the cells affect cell growth, HIT-T15 cells were electroporated with PURE-A, and growth and presence of $SNAP25_{197}$ were monitored for 10 days. Figure 2A demonstrates that the presence of the toxin delayed growth when compared to controls, but toxin-treated cells were able to replicate normally after a recovery period. Figures 2B and 2C demonstrate that cleavage of SNAP25 was detected by Western blot at all time points tested when PURE-A was introduced into the cells.

Having determined that HIT-T15 cells were an appropriate cloning host for cloning the BONT/A receptor, these cells were transfected with media from AmphoPack® 293 producer cells containing a retroviral particles. The retrovirus used to infect the HIT-T15 cells contained the pRetroLIB® vector from Clontech. The cDNA human brain library inserts were cloned at the stuffer fragment using restriction endonuclease Sfi sites. The entire insert of these constructs, from the 5'LTR to the 3'LTR, incorporates into the genomic DNA of the host HIT-T15 cell.

Cells were cultured for four extra days to allow expression of the cDNA packaged in the virus vector. Selection of individual cells expressing putative

BONT/A receptors was achieved by incubating the cells for 30 min at 4°C with PURE-A attached to magnetic beads (Dynabeads®, Dynal Biotech). Cells able to bind PURE-A were selected with a magnet, washed, and plated again in dishes. Individual colonies were formed in the dish, as shown in Figure 3, which were surrounded by magnetic beads.

Those colonies were picked and cultured in 96 well plates. When individual wells were close to confluence, cells were exposed to 1 nM PURE-A and inhibition of insulin release upon glucose stimulation was measured using an enzyme-linked immunoassay (Fig. 4). As shown in Figure 4, HIT-T15 cells at later passage numbers have a reduced production and secretion of insulin; this is the reason why in the figure shows that the isolated clones' level of insulin release is lower than in the control cells, which were used at a lower passage.

Representative clones C6 and C7 demonstrated a reduction in the amount of insulin release in high glucose medium (25 mM) after treatment with 1 nM PURE-A (Fig. 5). The percentage of release is calculated using the amount of insulin released by cells exposed to toxin divided by the amount of insulin released by cells which were not exposed to toxin.

Expanded cultures of clones C6 and C7 were also exposed to PURE-A; cells were lysed and Western blots

performed to verify toxin uptake by immunological detection of $SNAP25_{197}$ (Figure 6).

The genomic DNA of the C7 cell line was used as a template in PCR reactions. The PCR primers used were LIB 5'® and LIB 3'® from Clontech - these have the nucleotide sequences labeled SEQ ID NO: 1 and 2 above, respectively; these primers anneal to vector sequences just upstream and downstream, respectively, of the cloned insert region. The PCR reaction resulted in the amplification of a 12-15 kb amplicon (Figure 7A, lane 2).

We next performed a nested PCR reaction in an attempt to obtain smaller DNA fragments, as shown in Fig. 7B. Fragments were purified and cloned in Topo®-XL vectors (Invitrogen, Inc.). Plasmids containing inserts were partially sequenced, and analysis of the results demonstrated that the insert was derived from the retroviral vector.

The neuroblastoma cell line Neuro-2A possesses the high affinity uptake system for BoNT/A

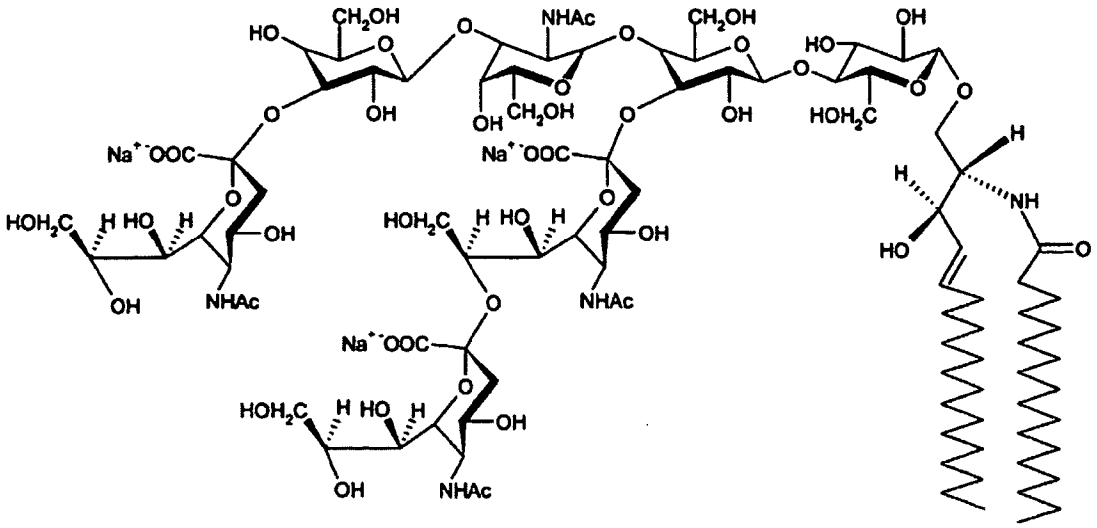
We screened neuronal cell lines, (cell lines Neuro 2A, SH-SY%Y, N1E-115, SK-N-BE(2), SK-N-DZ, SK-N-F1, BE(2)-C, SK-N-SH, NB4 1A3, BE(2)-M17, C1300, NG108-15, HCN-1A, HCN-2 TE 189.T, and ND8/34) as described above, for their ability to uptake BoNT/A in cell culture. For the first step of the screening, cells were exposed to 1nM BoNT/A (900 kDa) overnight, and Western blots using antibodies selective for the presence of $SNAP25_{197}$ were performed.

Three cells lines: Neuro-2A, SH-SY5Y, and NG108-15 displayed the highest levels of $SNAP\text{-}25_{197}$ following

BONT/A exposure (Fig.8) and were chosen for further evaluation.

The presence of the high affinity receptor is characterized by two attributes: rapid toxin uptake upon cell exposure, and the low toxin concentrations needed for intoxication. We evaluated the three selected cell lines for those criteria and discovered that Neuro-2A cells were able to take up toxin in less than 10 minutes, since a band for $SNAP25_{197}$ was seen ten minutes after treatment (Fig. 9A), the earliest time point tested. In contrast, the earliest point we detected the presence of $SNAP\text{-}25_{197}$ in SH-SY5Y cells was 6 hours (Fig. 9B).

Toxin uptake was also increased by pretreating the cells with the complex ganglioside GT1b. This naturally occurring sialoglycolipid, which can be purchased from a number of sources, including Alexis Biosciences Corporation, Ltd.) has the following structure:



Neuro-2A cells pretreated with ganglioside can be affected by toxin at concentrations as low as 12.5pM (Fig. 10). The ganglioside GT1b has been described as the sugar moiety to bind to the neurotoxin binding domain in conjunction with binding to the unknown protein receptor. These data led us to conclude that Neuro-2A cells contain the high affinity uptake system for toxin, and were subsequently used to purify the receptor for BoNT/A.

Strategy 2: Isolation of the BoNT/A receptor from Neuro-2A cells

Neuro-2A cells were used as the source for the isolation of the protein receptor which binds BONT/A, as this cell line binds the toxin with the highest affinity among the cell lines tested, and internalizes the toxin rapidly. For this work we used the trifunctional sulfo-SBED reagent described above as a cross linking reagent. This reagent, after first being linked to biotin, will biotinylate a target protein receptor, which can then be subsequently precipitated using avidin as the affinity ligand.

As shown in Figure 11, the reagent is first linked to PURE-A through reaction with an amine-containing group in the toxin molecule. Then cells were exposed to 5 nM PURE-A-sulfo-SBED complex at 4°C for 2-4 hours to avoid internalization of the toxin bound to receptor. Following binding of the receptor to the toxin, UV light was used to cross-link the neurotoxin and the receptor, and cells lysed.

The complex is isolated from the cell-free lysate using avidin beads and run on PAGE-SDS. Figure 12A shows the precipitated Neuro-2A protein visualized via silver stain. The SDS-PAGE was non-reduced, leaving the intact cross-linking reagent to bind PURE-A to putative receptor. The results show a large molecular protein complex (~250 kDa) that was isolated from cells

treated with BoNT/A, which would indicate the receptor has a size of approximately 100 kDa.

Figure 12B shows that the same sample was run on a reducing polyacrylamide gel to separate the toxin from the putative receptor. Following transfer of the reduced proteins to a membrane, the membrane was probed with antibodies to PURE-A. The resulting Western blot has two immunoreactive bands, which have molecular weights corresponding to the toxin (150 kDa) and heavy chain (100 kDa).

The membrane-transferred proteins from the reducing gel were also probed with antibodies selective for each of FGFR1, FGFR2, FGFR3, FGFR4, FGFR5 in an effort to more specifically identify the receptor to which the PURE A toxin bound. Figures 13A-D (corresponding respectively to FGF1, FGF2, FGF3, and FGF4) shows the resulting Western bots from these experiments. Only antibodies selective for FGF3 (Figure 13C) bound to bands in the Western blot. The main band has a molecular weight of approximately 80 KDa. However, higher molecular weight bands could also be detected, implying that the major band may be derived from a larger polypeptide.

We next surveyed a series of cell lines for the presence of the receptors FGFR1-4 in cell lysates. When indicated below, the presence of ganglioside GT1b was evaluated using an antibody to GT1b in fixed but non-

permebealized cells. The cells used were as follow: a) Neuro-2A cells, which contain a high affinity BONT/A receptor protein and ganglioside GT1b, b) SH-SY5Y cells which contain a low affinity BONT/A receptor, c) PC-12 cells, which contain a low affinity BONT/A receptor protein, and no ganglioside GT1b, d) HIT-T15 cells which contain ganglioside GT1b but no BONT/A receptor, e) HIT-T15 "C7" cells (obtained as explained above from exposure to retrovirus cDNA human brain library), which are able to take up toxin (see Figure 5 (inhibition of insulin release) and Figure 6 (cleavage of SNAP 25)), and thus implicitly contain a BONT/A receptor.

Cell-free lysates were made from each of these cell lines (Neuro-2A, SH-SY5Y, PC-12, HIT-T15, and HIT-T15 clone C7) and were evaluated for the presence of the FGF receptors FGFR1, FGFR2, FGFR3, and FGFR4 through Western blots. (See Figures 14A-D, respectively.)

The data from the Western blot revealed that FGFR2 and FGFR4 were not present in any of the cell lines. FGFR1 was present in all cell lines tested, including the ones that do not take BoNT/A, such as HIT-T15 (wt). FGFR3 was not present in the HIT-T15 wt cell line, but in contrast it was clearly present in the HIT-T15 C7 clone, generated from retroviral infection. This clone, upon expression of the cDNA inserted by the

retrovirus, is able to take up BoNT/A. The banding in HIT-T15 C7 was almost identical to that of Neuro-2A cells, but was different from the bands observed in SH-SY5Y cells that display low affinity BONT/A uptake.

To corroborate that BONT/A toxin enters Neuro-2A cells through the FGFR3 we performed a competition experiment with PURE-A at 5 nM concentration. Without added FGF, the toxin produces an almost complete cleavage of $SNAP25_{206}$ into $SNAP25_{197}$ at this concentration. We added FGF1 and FGF2 at different concentrations ranging from 0.1 nM (the FGF concentration that produces its characteristic biological effect) to 200 nM in order to be certain to obtain full competition. No ganglioside GT1b (other than that endogenous to the cell membranes of Neuro-2 cells) was added. The cells were exposed to either PURE A or PURE A plus FGF for 10 minutes. Table 1, which Applicants do not claim is a complete tabulation of FGF receptors and species, shows certain members of the family of FGFRs and their known ligands and tissue distribution.

NAME	FGFR1		FGFR2		FGFR3		FGFR4	FGFR5
Variant	IIIb	IIIc	IIIb	IIIc	IIIb	IIIc		
Ligands	FGF-1	FGF-1 FGF-2 FGF-4	FGF-1 FGF-2 FGF-7	FGF-1 FGF-2 FGF-4	FGF-1 FGF-2	FGF-1 FGF-4 FGF-9	FGF-1 FGF-2	FGF-1 FGF-2
Tissues	Brain, bone, kidney, skin, lung, heart, muscle, neuron		Brain, kidney, skin, lung, liver, glial cells		Brain, kidney, skin, lung		Lung, liver, kidney	Brain, skin, lung,testis

Table 1: FGFR variants, ligands, and tissue distribution obtained from Sigma RBI Cell Signaling and Neuroscience Catalog, 2003 (hereby incorporated by reference herein)

Figure 15 shows the results of a Western blot of the receptor competition experiment in Neuro-2A cells of PURE-A and either FGF-1, FGF-2, or both FGF-1 and FGF-2. Neuro-2 cells were incubated with toxin at 37°C and a concentration of 5 nM, with and without increasing concentrations of either or both FGF species. Cells were then harvested, lysed and 30 μg of protein per sample were subjected to electrophoresis in a 12% Bis-Tris polyacrylamide gel. Following transfer of the protein bands to a membrane, the membrane was probed with SMI-81 antibody that recognizes uncleaved and cleaved SNAP25. Similar experiments were performed for FGF-9, and the results were similar.

The competition experiment demonstrated that BoNT/A and FGF-1, FGF-2, and FGF-9 bind to the same receptor in the membrane of Neuro-2A cells. PURE-A at 5 nM without added FGF produces complete cleavage of SNAP25 to $SNAP25_{197}$. It has been reported that FGF produces its physiological effects on cells at 0.1 nM, but at this concentration FGF-1 or FGF-2 do not compete with BONT/A for receptor binding.

This result suggests that the biochemical pathway triggered by the previously characterized biological activity of FGF on cells is not sufficient to displace

BONT/A when bound to its receptor. Competition between FGF1 or FGF2 is first observed at an FGF concentration of 5 nM, that is, at an equimolar concentration of toxin and FGF. At a 5 nM concentration of either or both FGHF1 and FGF2 cleavage of SNAP25 is reduced to about 50%. When the FGF concentration was increased to 200 nM full competition for toxin binding was achieved and all observable SNAP25 was present in its uncleaved form $SNAP25_{207}$.

These data demonstrate that FGFR is a pathway for toxin binding and internalization. This conclusion is supported by the evidence presented here, and in light thereof, is supported by previous individual findings. For example, Schengrund et al., 29 Brain Res. Bull.917-924 (1992) demonstrated that ^{125}I-BoNT/A binds preferentially to a protein of ≅ 80 kDa isolated from brain synaptosomes, and to a lesser extend to a protein of ≅ 116 kDa from the same extracts. These molecular weights correlate well with the bands observed in our Western blots for FGFR3. The receptor for BoNT/A is probably a glycoprotein, since binding to both the protein receptor and to the ganglioside (GT1b in the case of BoNT/A) are needed for high affinity toxin binding and internalization into neurons. In addition, the C-terminal portion of the heavy chain, that is the region involved in binding to the protein receptor, is structurally similar to FGFs.

The FGF receptors interact with complex gangliosides such as GT1b at the plasma membrane, and the activity of the receptor is modulated by these interactions (Miljan and Bremer, Science's STKE (http://stke.sciencemag.org/cgi/content/full/sigtrans%3b2002/150/re15) November 26, 2002). In other systems gangliosides have been observed to modulate ligand binding, regulate receptor dimerization/polymerization, receptor activation state and subcellular localization. Neuronal cells that take up BONT/A express GT1b, and moreover, FGFR3 is expressed in spinal cord neurons during development and in motoneurons (Dono, R., 284 Am. J. Physiol. Regul. Integr. Comp. Physiol. R867-R881 (2003)). In vitro, FGFR3 ligands such as FGF2 and FGF9 promote survival of spinal motoneurons in culture, and upregulate the enzyme choline acetyltransferase. Interestingly, the binding of FGF to its receptor and to BoNT/A each increase serotonin uptake by neural cells in culture (Pelliccioni et al., 17 Mol. Neurosci. 303-310 (2002).

Upon ligand binding and internalization the ligand:FGFR complexes begin their passage through endosomal compartments. The intravesicular pH drops along the endocytic pathway; this lowering of pH causes dissociation of ligand-receptor complexes and permits recycling of ligand-free receptors (Sorkin, 3 Frontiers in Bioscience d729-738 (1998). Similarly, following binding the toxin is internalized into endosomes and after acidification of the endosome the translocation

domain (N-terminus of the heavy chain) undergoes a conformational change that facilitates delivery of the light chain to the cytoplasm. *See* Schiavo et al. (previously cited), Schmid et al., 394 Nature 827-830 (1993); Koriazova et al., 10 *Nat. Struct. Biol.* 13-18(2003). The FGFR ligand FGF-1 is known to be capable of crossing cellular membranes to reach the cytosol and the nucleus, and this translocation process is dependent on the maintenance of vesicle transmembrane potential (Malecki et al., 21 EMBO J. 4480-4490 (2002)).

Bafilomycin A1, a specific inhibitor of vacuolar proton pumps, blocked FGF-1 translocation completely, *id.*, and has also being described to block BoNT/A translocation to the cytoplasm from endocytic vesicles (Keller, Naunyn Schmiedebergs Arch Pharmacol 365(Suppl 2):R26 ABS 69 (June 2002).

This information by itself does not suggest the invention claimed herein. However, when combined with our data the cited literature demonstrate that FGFR (in the case of BONT/A, FGFR-3) functions as a selective receptor for toxin uptake and internalization.

The following examples are exemplary only and do not limit the claims, which are defined solely by their terms.

Example 1:

A fusion protein comprising the C terminal portion of the heavy chain of BONT and the light chain of BONT/E is tested for its ability to selectively bind and intoxicate BONT/A susceptible cells. A preparation comprising dilutions of the fusion protein is incubated with HIT-T15 insulinoma cells expressing exogenous FGFR3 in the presence of GT1b ganglioside. The ability of the fusion peptide to bind and enter the insulinoma cells is detected by detecting secretion of insulin in response to the presence of glucose, as described above. By contrast, insulin secretion is unaffected in cells not expressing FGFR3.

The results of this assay show that amount of insulin secreted into the culture medium is decreased in a dose-dependent manner when the fusion protein is added to the culture medium. Western blots of cell lysates will show the conversion of full length SNAP-25 to the cleaved form typical of the proteolytic activity of the BONT/E light chain protease. This assay therefore is useful in showing that the fusion peptide is able to bind and enter BONT/A susceptible cells.

The same fusion protein is capable of intoxicating cells of the neuromuscular junction.

Example 2:

A fusion protein comprising the receptor binding portion of an FGF species capable of binding FGFR3 (including FGF1, FGF2, FGF4 and FGF9) and the translocation domain and light chain of BONT/E is tested for its ability to selectively bind and intoxicate BONT/A susceptible cells. The assay is conducted as described in Example 1 above, with similar results; the detected cleaved SNAP-25 fragments are characteristic of BONT/A intoxication.

Example 3:

BONT/A, produced from fermentation of Clostridium botulinum is produced using standard fermentation techniques. Either or both the bulk preparation and purified, formulated versions of expressed toxin are tested for purity and activity as follows. A preparation comprising dilutions of the BONT/A preparation is incubated with HIT-T15 insulinoma cells expressing exogenous FGFR3 in the presence of GT1b ganglioside. The ability of the toxin to bind and enter the insulinoma cells is detected by detecting secretion of insulin in response to the presence of glucose, as described above. The specific activity of the preparation can be calculated from the determined protein concentration and the activity of the preparation at various doses.

These data are submitted to the U.S. Food and Drug Administration by a pharmaceutical company as part of data demonstrating how BONT/A is manufactured and tested. This information is considered by the FDA, who decides to permit the manufacture and sale of this lot of BONT/A, and subsequent lots made and tested in a similar manner, as a therapeutic pharmaceutical product based in part on this bulk and/or formulation assay data.

The pharmaceutical comprising the BONT/A is then offered for sale as a prescription medication.

Example 4:

Same as Example 3, however the polypeptide produced is the fusion neurotoxin of Example 1, produced in *E. coli*. Both bulk and/or formulation lots of the fusion neurotoxin are tested as indicated above, the data submitted to the FDA, and a decision to grant marketing approval, or continued sales of such fusion polypeptide as a therapeutic agent, is made by the FDA based at least in part on such data. The pharmaceutical company then offers the fusion neurotoxin for sale as a prescription therapeutic agent.

Example 5:

An *in vitro* assay is established using cloned FGFR3 bound to a solid support in the presence of ganglioside GT1b. The bound FGFR3 is first saturated with BONT/A heavy chain (H chain) in phosphate buffered saline (PBS), and washed free of unbound FGF. A test compound from a combinatorial library of compounds is contacted with the receptor under substantially physiological conditions (e.g., PBS), and the eluate collected. The H chain concentration in the eluate is compared to the H chain concentration of a control eluate in which H chain was not first bound to FGFR3.

Test compounds which are able to strongly bind FGFR3 and compete with H chain for FGFR3 binding (for example, by the method described in this section) are candidates compounds for the development of an antidote to acute botulism poisoning.

The examples provided herein are simply illustrations of various aspects of the invention, which is to be understood to be defined solely by the claims which follow this specification. All citations given here are hereby incorporated by reference herein.

CLAIMS

What is claimed is:

1) A method of screening a sample for the presence of an agent able to compete with BONT/A neurotoxin for selective binding to cells susceptible to BONT/A intoxication comprising:

a. contacting said sample with a composition comprising FGFR3 receptor and optionally GT1b ganglioside, and

b. determining whether said molecule selectively binds FGFR3,

wherein selective binding of said molecule to FGFR3 indicates that said molecule is able to selectively compete with BONT/A neurotoxin for selective binding to cells susceptible to BONT/A intoxication, and wherein if said agent is BONT/A, said method does not comprise an LD_{50} assay.

2) The method of claim 1 wherein the FGFR3 is expressed on the surface of a cell.

3) The method of claim 2 wherein said cell is comprised in a tissue of a living animal.

4) The method of claim 1 wherein said contacting step is performed *in vitro*.

5) The method of any one of claims 1-4 wherein said agent is BONT/A.

6) The method of any one of claims 1-4 wherein said agent is not BONT/A.

7) The method of claim 6 wherein said agent comprises a receptor binding domain of a heavy chain of BONT/A.

8) The method of claim 6 wherein said agent comprises a receptor binding domain of an anti-FGFR3 antibody.

9) The method of claim 6 wherein said agent comprises a receptor binding domain of an FGF.

10) The method of claim 9 wherein said FGF is selected from FGF 1, FGF2, FGF4 and FGF9.

11) A method of determining the activity of a preparation of BONT/A comprising the method of claim 1.

12) The method of any one of claims 1-4 or claim 1 wherein said agent comprises an protease domain which cleaves a SNARE protein at a site other than that cleaved by BONT/A light chain.

13) The method of claim 12 wherein said protease domain comprises the active site of the light chain of a clostridial neurotoxin other than BONT/A.

14) The method of claim 13 wherein said protease domain comprises the active site of the light chain of BONT/E.

15) A method of inhibiting the activity of BONT/A in a human comprising administering to said human a first composition comprising an agent selected from the group consisting of FGFR3 and an FGF.

16) The method of claim 6 further comprising administering to said human a ganglioside GT1b.

17) A polypeptide able to compete with BONT/A for selective cell surface binding which is produced from a preparation in which the ability of said polypeptide to selectively bind to BONT/A-susceptible cells is assayed using a method comprising the steps: contacting said polypeptide with a composition comprising FGFR3 receptor and optionally GT1b ganglioside, and directly or indirectly determining whether said polypeptide selectively binds FGFR3, wherein ability to compete with BONT/A for cell surface

binding is indicated if said polypeptide selectively binds said FGFR3 receptor.

18) The polypeptide of claim 17 which comprises a cell surface binding domain of a BONT/A neurotoxin.

19) The polypeptide of claim 18 which comprises BONT/A.

20) The polypeptide of claim 18 which comprises a chimeric neurotoxin comprising a domain other than said cell surface binding domain which is derived from a neurotoxin other than BONT/A.

21) The polypeptide of claim 20 which comprises a proteolytic domain derived from BONT/E.

22) The polypeptide of claim 17 wherein said polypeptide is contacted with a receptor expressed on the surface of a cell.

23) The polypeptide of claim 22 wherein said ability to compete with BONT/A for cell surface binding is indirectly determined by detecting cleavage of a SNARE protein.

24) The polypeptide of claim 17 wherein said preparation is a formulated human therapeutic drug.

25) The polypeptide of claim 17 wherein said preparation is made from a cell lysate or culture medium.

26) A polypeptide comprising BONT/A which is produced from a preparation in which the ability of said polypeptide to selectively bind to BONT/A-susceptible cells is assayed using a method comprising the steps: contacting said polypeptide with a composition comprising FGFR3 receptor and optionally GT1b ganglioside, and directly or indirectly determining whether said polypeptide selectively binds FGFR3, wherein the activity of BONT/A is said preparation is directly or indirectly determined by its ability to bind FGFR3.

27) The polypeptide of claim 26 which comprises a cell surface binding domain of a BONT/A neurotoxin.

28) The polypeptide of claim 27 which comprises BONT/A.

29) The polypeptide of claim 27 which comprises a chimeric neurotoxin comprising a domain other than said cell surface binding domain which is derived from a neurotoxin other than BONT/A.

30) The polypeptide of claim 29 which comprises a proteolytic domain derived from BONT/E.

31) The polypeptide of claim 26 wherein said polypeptide is contacted with a receptor expressed on the surface of a cell.

32) The polypeptide of claim 31 wherein the activity of said BONT/A is further determined by detecting cleavage of a SNARE protein.

33) The polypeptide of claim 17 wherein said preparation is a formulated human therapeutic drug.

34) The polypeptide of claim 17 wherein said preparation is made from a cell lysate or culture medium.

35) The polypeptide of claim 26 in which said said ability to compete with BONT/A for cell surface binding is determined *in vitro*.

36) The method of claim 26 wherein said BONT/A neurotoxin comprises a native BONT/A molecule.

37) The method of claim 36 wherein said BONT/A molecule is isolated from a *Clostridium botulinum* strain selected from the group consisting of: CL138, 137, 129, 13, 42N, Hall

A-hyper, 667Ab, NCTC 2916, P10845, Allergan-Hall A, 62A, Kyoto-F, type A NIH, NCTC 7272, 7I03-H, and Kumgo.

38) A method of marketing a neurotoxin selectively binding to the same binding site as does BONT/A comprising obtaining marketing approval from a governmental or regional regulatory authority for a therapeutic neurotoxin that is first produced from a bulk preparation, wherein said neurotoxin is assayed for specific binding to neural cells using a method comprising the steps: contacting said neurotoxin molecule with a composition comprising FGFR3 receptor and GT1b ganglioside and determining whether said neurotoxin molecule selectively binds FGFR3, packaging said neurotoxin for sale in a manner consistent with the requirements of said regulatory authority, and selling said neurotoxin.

39) The method of claim 37 wherein said BONT/A neurotoxin comprises a native BONT/A molecule.

40) The method of claim 38 wherein said BONT/A molecule is isolated from a *Clostridium botulinum* serotype A strain selected from the group consisting of: CL138, 137, 129, 13, 42N, Hall A-hyper, 667Ab, NCTC 2916, P10845,

Allergan-Hall A, 62A, Kyoto-F, type A NIH, NCTC 7272, 7I03-H, and Kumgo.

41) The method of claim 38 wherein the FGFR3 is expressed on the surface of a cell.

42) The method of claim 41 wherein said cell is comprised in a tissue of a living animal.

43) The method of claim 1 wherein said contacting step is performed *in vitro*.

44) The method of claim 38 wherein said neurotoxin is not BONT/A.

45) The method of claim 44 wherein said neurotoxin comprises a receptor binding domain of a heavy chain of BONT/A.

46) The method of claim 44 wherein said neurotoxin comprises a receptor binding domain of an anti-FGFR3 antibody.

47) The method of claim 44 wherein said neurotoxin comprises a receptor binding domain of an FGF.

48) The method of claim 47 wherein said FGF is selected from FGF 1, FGF2, FGF4 and FGF9.

49) The method of claim 44 wherein said neurotoxin comprises a protease domain which cleaves a SNARE protein at a site other than that cleaved by BONT/A light chain.

50) The method of claim 49 wherein said protease domain comprises the active site of the light chain of a clostridial neurotoxin other than BONT/A.

51) The method of claim 50 wherein said protease domain comprises the active site of the light chain of BONT/E.

52) A method of inhibiting the activity of BONT/A in a human comprising administering to said human a first composition comprising an agent selected from the group consisting of FGFR3, and anti FGFR3 antibody and an FGF.

53) The method of claim 6 further comprising administering to said human a ganglioside GT1b.

54) The method of claim 52 in which said agent is an anti FGFR3 antibody.

54) A method of screening a compound for the ability to bind to cells selectively targeted by BONT/A neurotoxin, comprising the steps contacting said compound with a composition

comprising FGFR3 receptor and GT1b ganglioside, and determining whether said compound selectively binds FGFR3, wherein selective binding of said compound to FGFR3 indicates that said molecule is able to bind to cells selectively targeted by BONT/A neurotoxin.

55) The method of claim 54 wherein the FGFR3 is expressed on the surface of a cell.

56) The method of claim 54 wherein said contacting step is performed *in vitro*.

57) The method of any one of claims 55 and 56 wherein said molecule is BONT/A.

58) A method of rendering a cell susceptible to cleavage of intracellular SNARE proteins by a neurotoxin, comprising inducing said cell to express FGFR3.

59) The method of claim 58 wherein said induction step is accomplished by transfecting said cell with an expression vector encoding FGFR3.

60) The method of claim 59 wherein said cell is a pancreatic acinar cell.

61) The method of claim 59 wherein said cell is a sensory neuron.

62) The method of any one of claims 58-61 wherein said induction occurs in vivo.

ABSTRACT

Methods and compositions for screening ligands to clostridial toxin receptors. Also disclosed are methods of assaying bulk preparations of such toxin, and for marketing toxin so assayed.

FIGURE 1

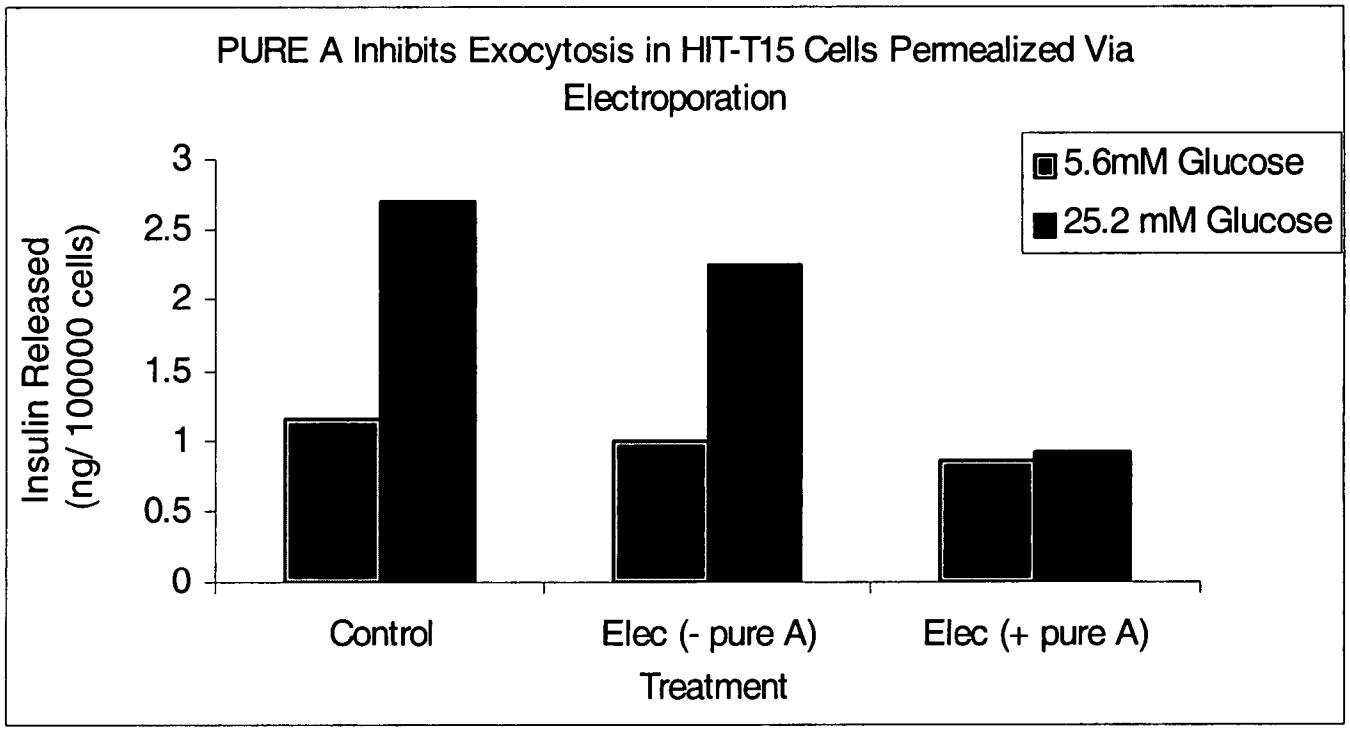
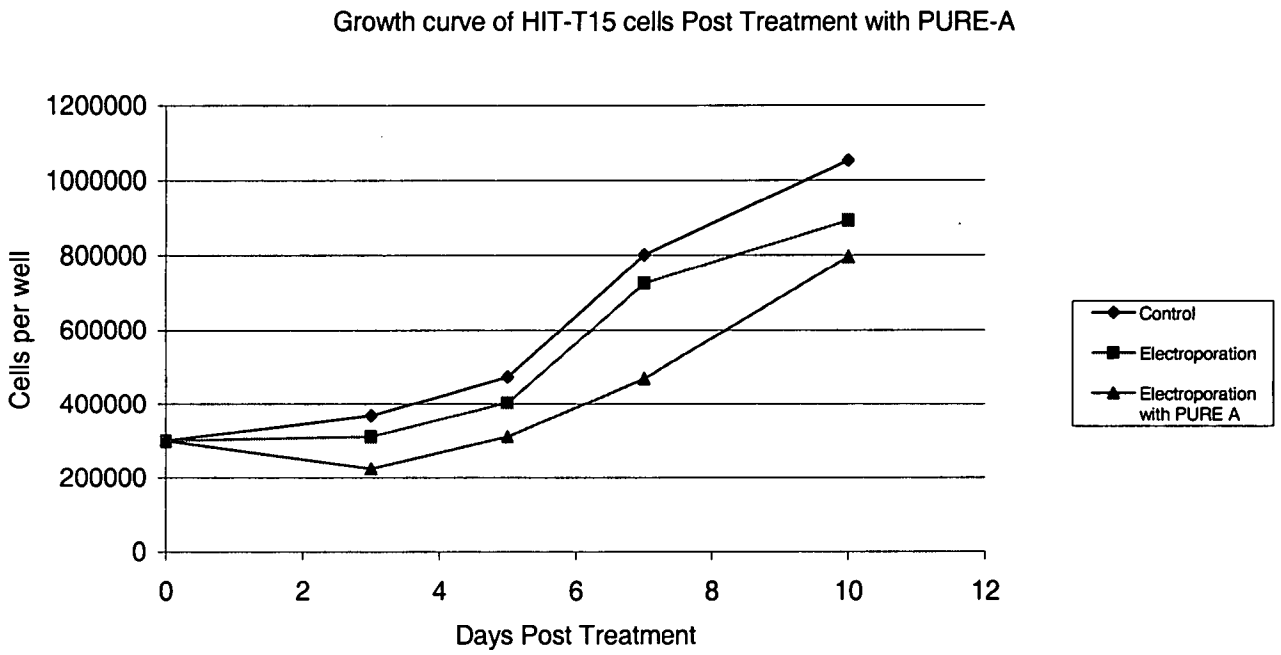

PURE A Inhibits Exocytosis in HIT-T15 Cells Permealized Via Electroporation
Insulin Released (ng/ 100000 cells)
3
2.5
2
1.5
1
0.5
0
5.6mM Glucose
25.2 mM Glucose
Control
Elec (- pure A)
Elec (+ pure A)
Treatment

FIGURE 2A


Growth curve of HIT-T15 cells Post Treatment with PURE-A
Cells per well
1200000
1000000
800000
600000
400000
200000
0
0
2
4
6
8
10
12
Days Post Treatment
Control
Electroporation
Electroporation with PURE A

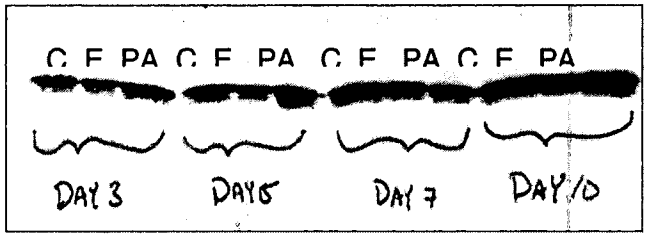


Figure 2B

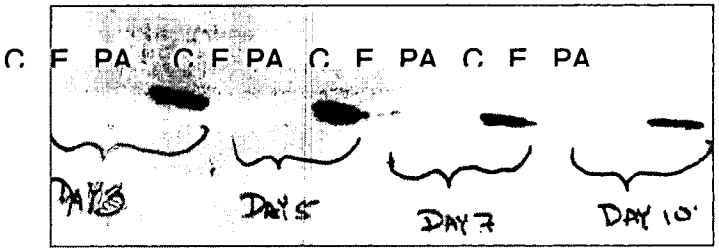


Figure 2C

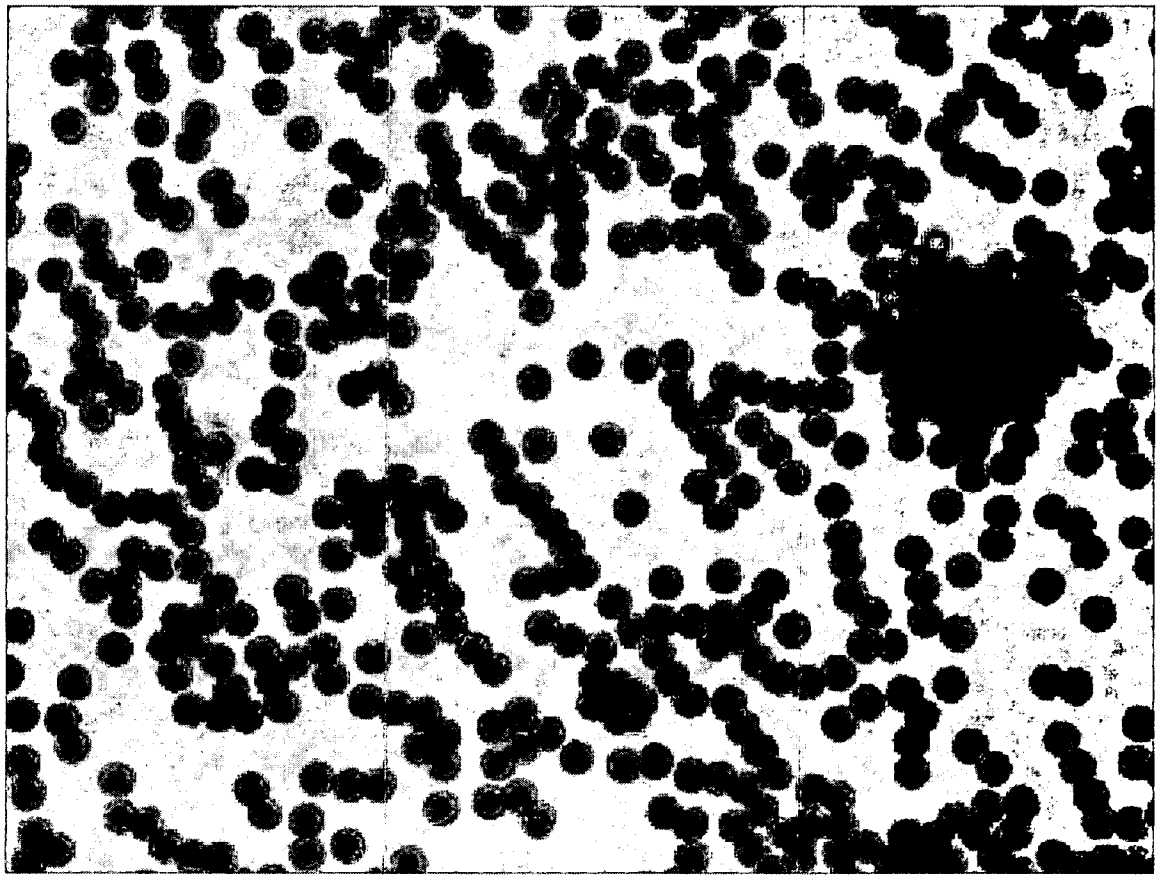


Figure 3

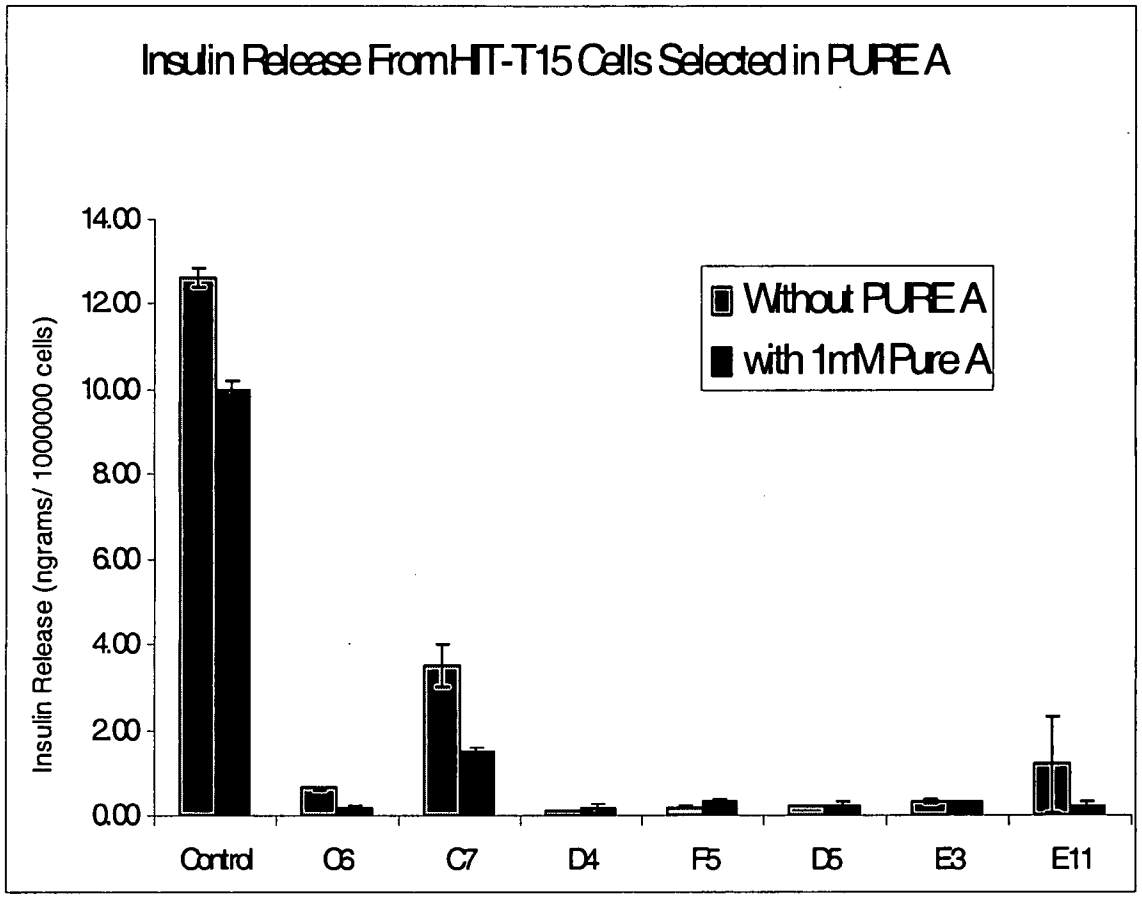


Figure4: Inhibition of insulin exocytosis caused by exogenous PURE-A on HIT-T15 cell lines. All cell lines except for control cells were previously exposed to virus containing human cDNA brain library. Cells were exposed to 1nM PURE-A. Insulin release was induced by high glucose media and was measured using an ELISA kit from Peninsula Laboratories.

Figure 4

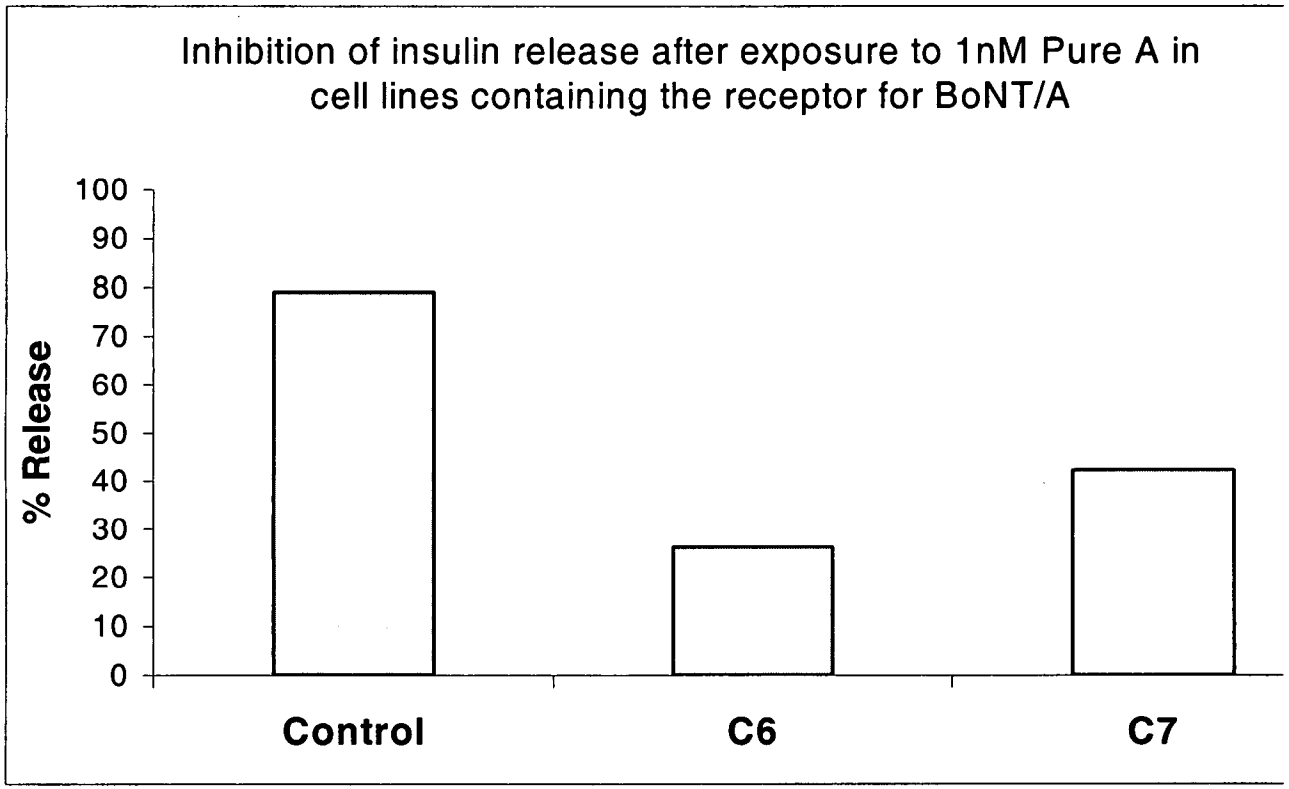

Inhibition of insulin release after exposure to 1nM Pure A in cell lines containing the receptor for BoNT/A
% Release
100
90
80
70
60
50
40
30
20
10
0
Control
C6
C7

Figure 5

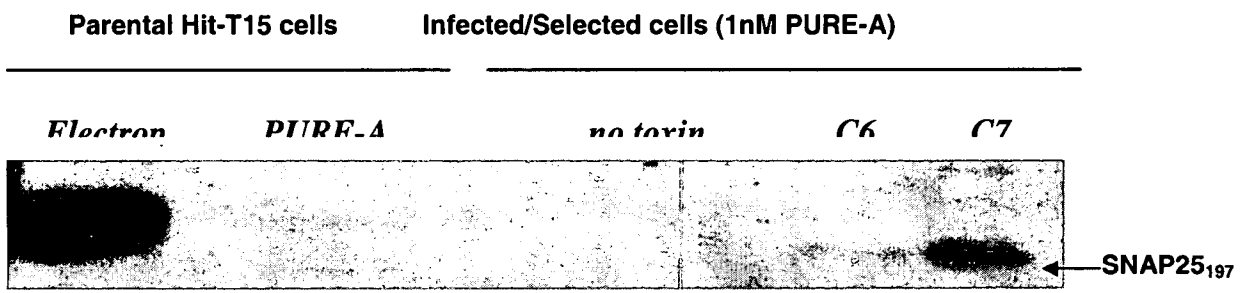

Parental Hit-T15 cells
Infected/Selected cells (1nM PURE-A)
Electron
PURE-A
no toxin
C6
C7
SNAP25197

Figure 6

Figure 7: A. PCR product obtained using genomic DNA from HIT-T15 C7 cell line as a template and the LIB 5′ and LIB 3′ primer pair. A band of 12 kDa was obtained (arrow). The band was purified and a nested PCR was performed using **WHICH???** other primer pairs. **B.** Bands of several molecular weights were obtained from the nested PCR experiment. The polynucleotide bands were purified from the gel and cloned in Topo-XL vectors for subsequent sequencing.

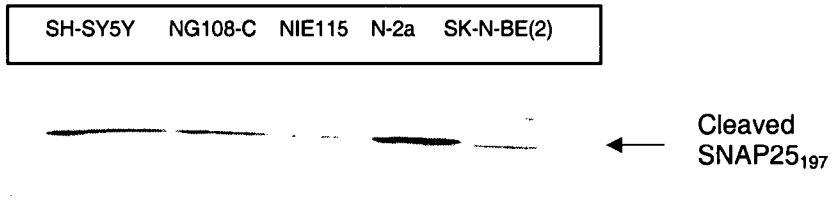
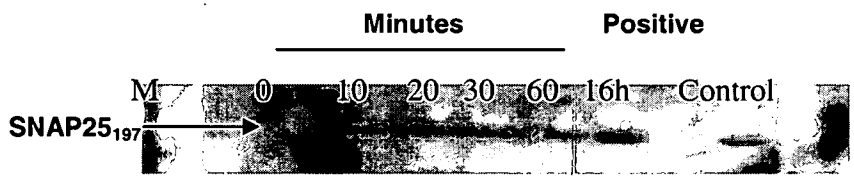


Figure 8: Western blot of 5 cell lines treated with 1 nM of Pure BoNT/A over night. Same amount of protein loaded per lane.

A.



B.

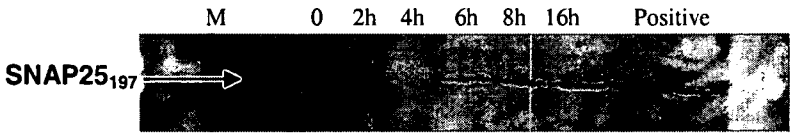


Figure 9: A. Time Course of SNAP25 cleavage in Neuro-2A cells treated with 1nM BoNT/A complex analyzed by Western Blot with an antibody specific for $SNAP25_{197}$ (different amounts of protein loaded per lane). **B.** Time Course of SNAP25 cleavage in SH-SY5Y cells treated with 1nM BoNT/A complex analyzed by Western Blot as in A.

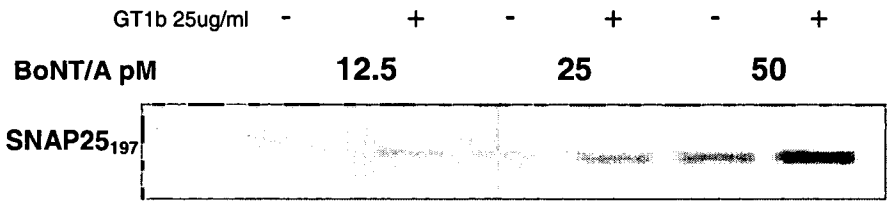


Figure 10: Neuro-2a cells pre-treated with GT1b and expose to pM concentrations of toxin. 140 µg of total protein was loaded per lane.

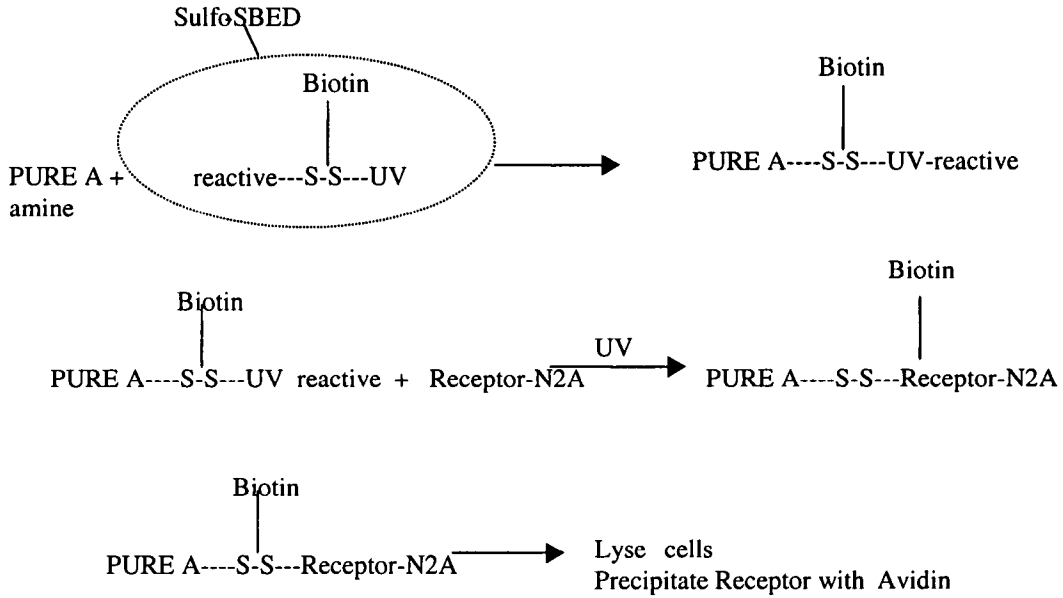


Figure 11: Overall reaction scheme to crosslink Pure A to a Putative Receptor on Neuro-2A cells (Receptor-N2A) using the cross-linking reagent sulfo-BED

FIGURE 12

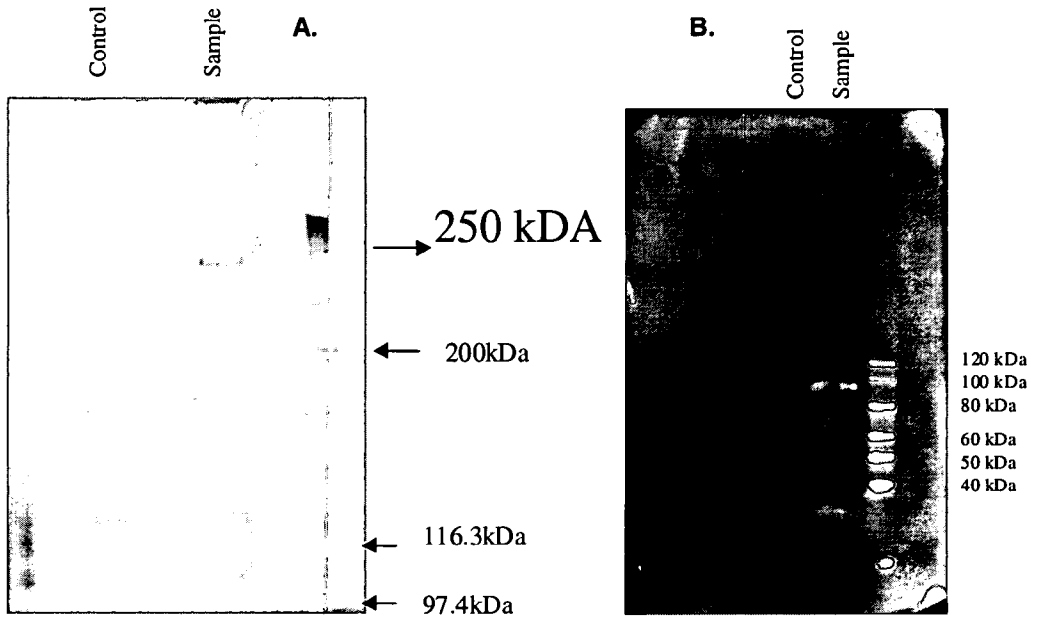

Control
Sample
A.
250 kDA
200kDa
116.3kDa
97.4kDa
B.
Control
Sample
120 kDa
100 kDa
80 kDa
60 kDa
50 kDa
40 kDa
α-Toxin

Figure 13

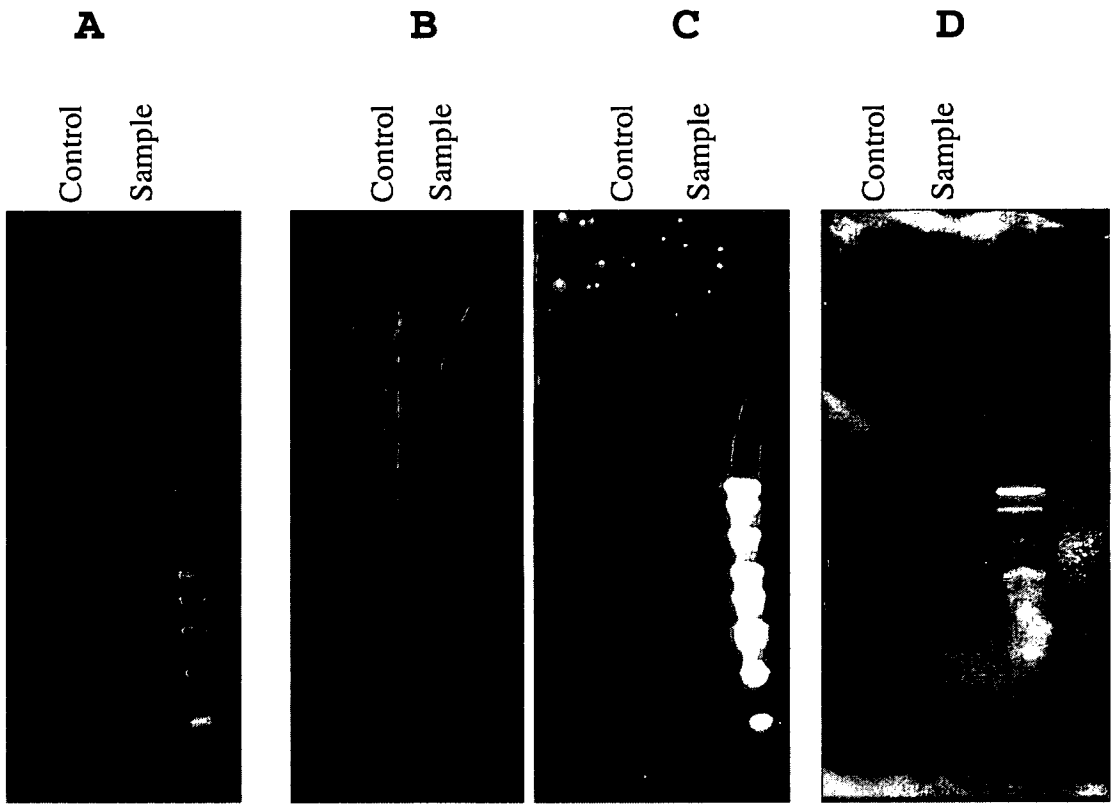


Figure 13: Western blot of the toxin-receptor complex in a reducing PAGE-SDS probed with antibodies to FGFR1-4, respectively. Only antibodies to FGFR3 showed immunoreactive bands in the Neuro-2A sample.

FIGURE 14

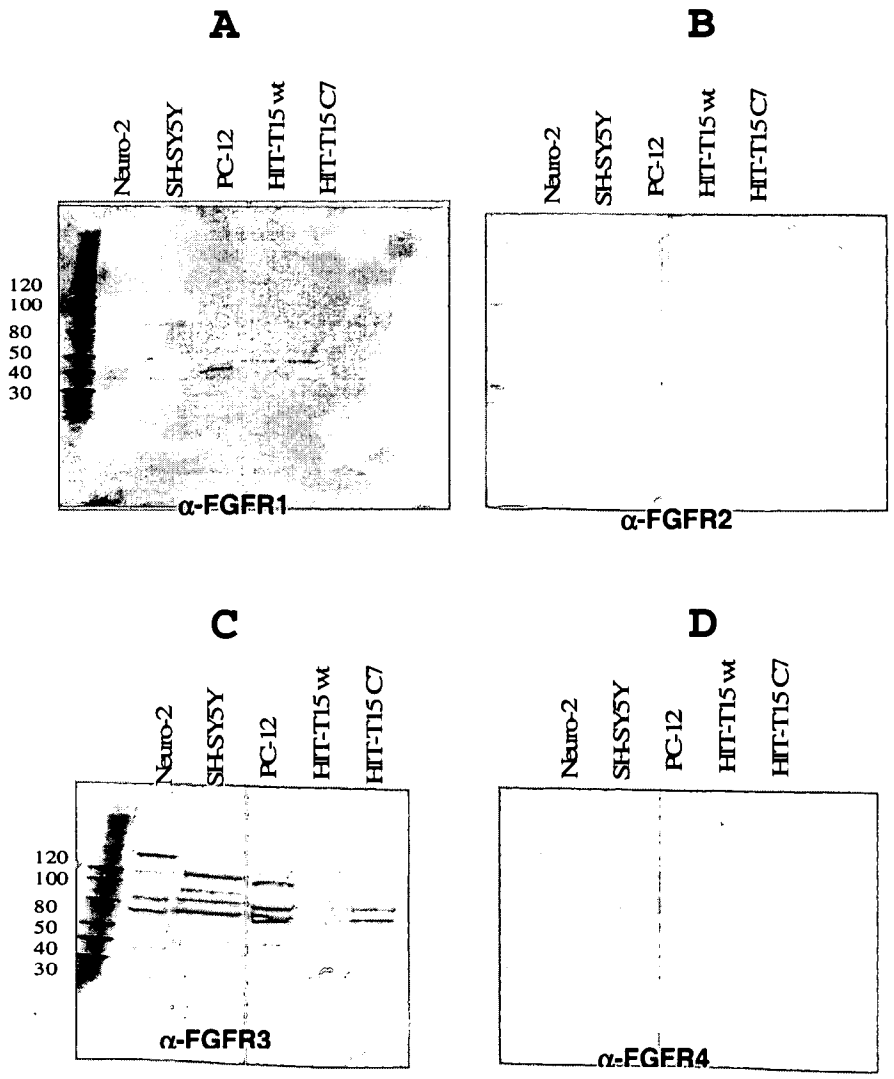

A
B
C
D
Neuro-2
SH-SY5Y
PC-12
HIT-T15 wt
HIT-T15 C7
120
100
80
50
40
30
α-FGFR1
α-FGFR2
α-FGFR3
α-FGFR4

FIGURE 15

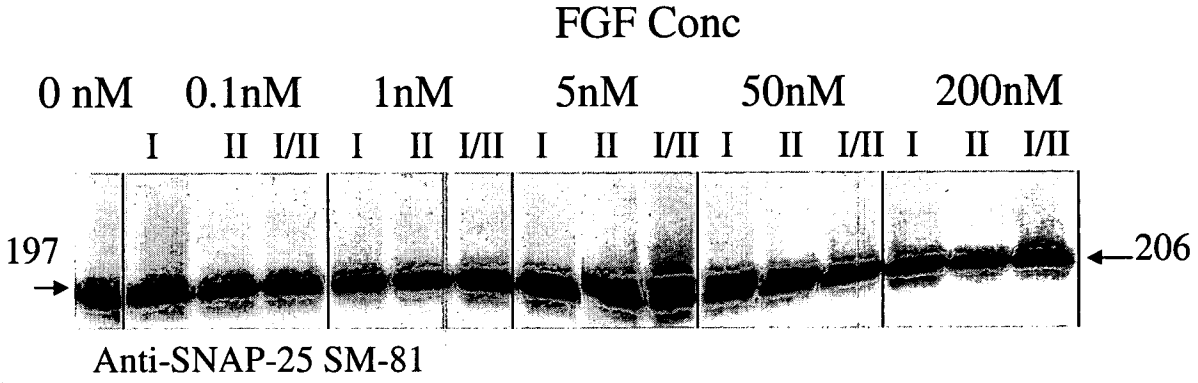


Figure 15: Western blot of the competition experiment of Pure A and FGF. Toxin at 5 nM and increasing concentrations of FGF were added together to cells for 10 min at 37°C. Following electrophoresis, samples were probed in a Western blot with SMI-81 antibody, which recognizes uncleaved and cleaved SNAP25.

Figure 16A

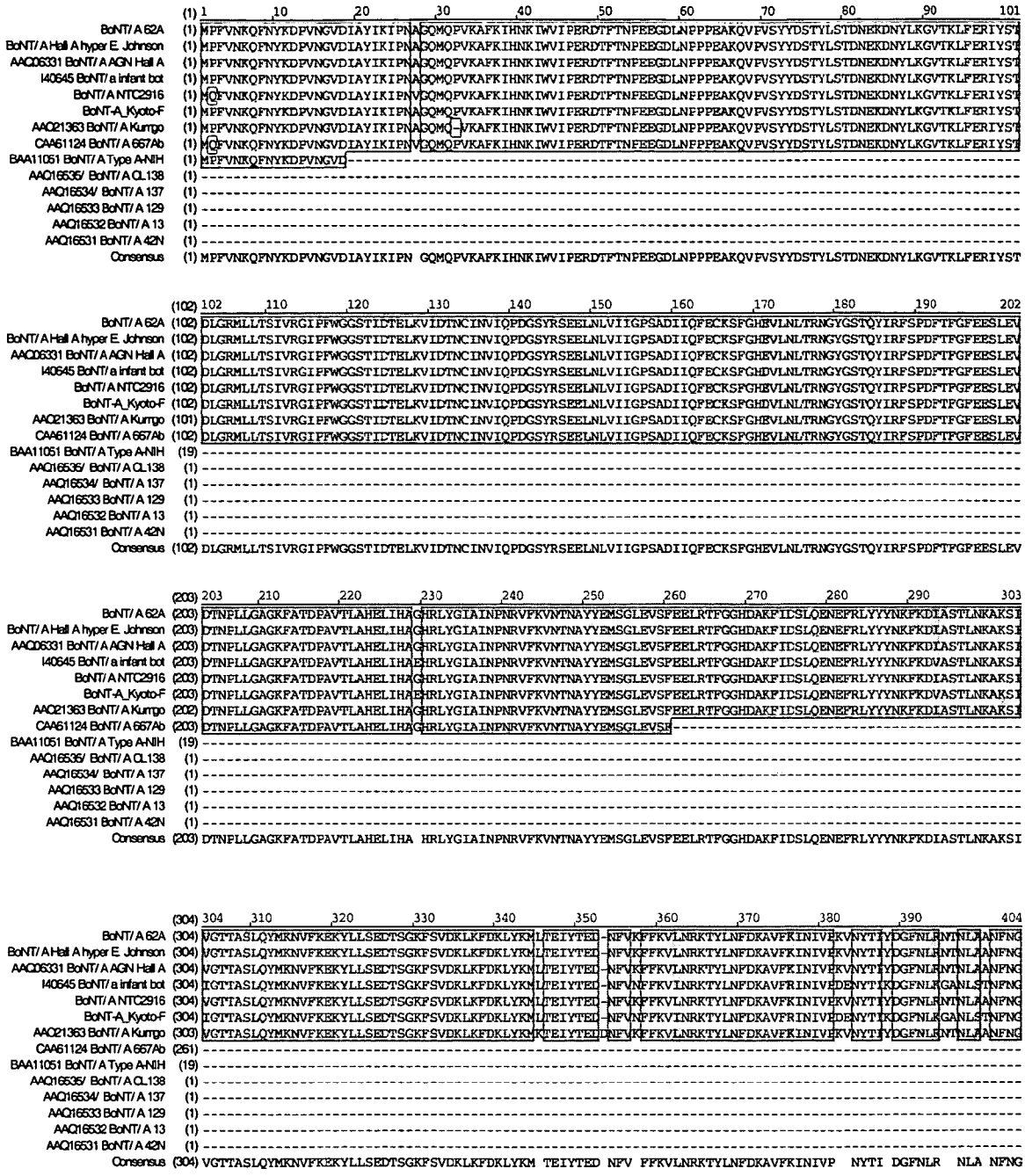


Figure 16B

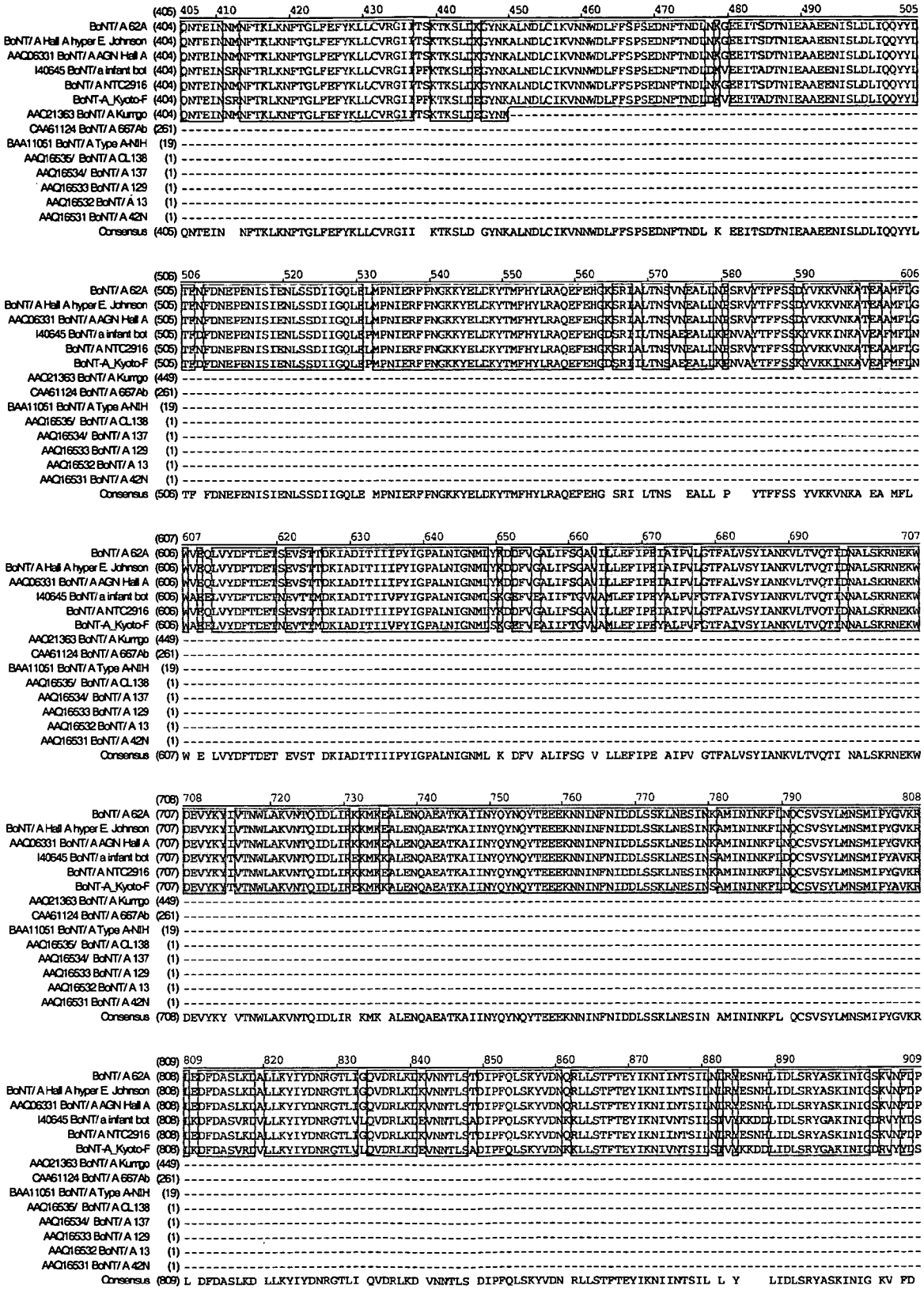


FIGURE 16C